



Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project
licences granted January – June 2020



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1. Affect and cognition in rodents

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Animal types	Life stages
Rats	juvenile, adult, aged
Mice	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 is the aim of this project?

To identify and characterise the psychological and biological mechanisms underpinning the affective and cognitive processing of rewarding and punishing stimuli and dysfunctions/disturbances of such processes.

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

Why is it important to undertake this work?

Psychiatric disorders are a major health burden in the UK (and worldwide). One bottleneck in developing novel treatments is a lack of targets for drug discovery. This project will develop the understanding of emotion and cognitive function in rodents, and how these fail in rodent models of psychiatric disorders. In particular, the project will characterise the relationship between affective, motivational, and cognitive processing of rewarding and



punishing stimuli. This knowledge will be used to determine how biological (e.g. variation in risk genes for particular disorders) or environmental (e.g. early life stress) risk factors are related to specific aspects of the affective and cognitive processing of rewarding and punishing stimuli; and illuminate the biological pathways mechanisms linking these risk factors to their behavioural effects. Such outputs will aid the drug development process by identifying targets for new therapeutic techniques.

The psychological and biological mechanisms underpinning affective and cognitive processes are also of particular interest in the domain of lab-animal welfare. Many thousands of rodents are used annually in laboratory work based involving techniques such as injections. The welfare impact of these techniques - in particular the degree to which they disrupt normal cognitive and affective function - has received relatively little direct scientific investigation. Thus in many cases, the aversive impacts, and means to minimise them, are not known. This project will provide information about how aversive some common techniques are and about ways to minimise or avoid that harm.

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

The primary outputs from the project will be new information. This will typically communicated by scientific publication or presentation (but also engagement with non-specialist audiences).

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short-term, the primary benefit will be to other academic and industrial scientists working in the field. Previous work has been well cited - indicating its use by others. In addition, I am part of ongoing collaborations with representatives of the pharmacological industry that were established in part due to work undertaken on previous projects - suggesting that the results from the current project will also be of benefit to industry.

In the longer-term, the cumulative gains in knowledge will support application and translational extension. For example, some of the work to be performed on this project concerns key risk-genes for psychiatric disorders. Understanding the function of such genes could contribute to the development of novel therapeutic strategies.

How will you maximise the outputs of your work?

I am involved in a number of academic collaborations within the field (across several institutions within and outside the UK) and outside the field (relating to the application of hedonic assessment methods to pig production). In addition, I have ongoing collaborations with the pharmaceutical industry. These collaborations will continue and will support the impact of the current work.

In addition, I have a strong track-record of academic publication and conference dissemination (including the organisation of an annual conference on associative learning and neuroscience). This will continue across the life of the current project - supporting the wide dissemination of the outputs of the current work.

Finally, I am committed to open science (including the dissemination of reliable null results) - which will ensure that a fully representative impression of the outputs of the current work will be widely available.



Species and numbers of animals expected to be used

- Rats: 1400
- Mice: 200

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

A maximum of 1600 animals will be used across the life of the project. The bulk of the experiments will use rewarding stimuli (its primary purpose is the investigation of the mechanisms involved in processing rewards, and dysfunction in these mechanisms in models of risk factors for psychiatric disorders), but a subset (approximately 25%) of experiments will involve aversive stimuli (e.g. pain from foodshock or nausea from LiCl injection). Approximately 12.5% of the animals will also receive surgical implantation of an oral cannula under anaesthesia and approximately 12.5% will receive direct exposure to stress-inducing stimuli/events. The typical duration of experiments will be 3-6 months, however for aged animals (e.g. experiments relating to dementia or aging) animals may be in the laboratory for up to 24 months (although this will typically involve only 3-6 months of direct experimental work).

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The bulk of procedures will produce (at most) transient pain or distress, with minor disturbance of normal behaviour. Extended or excessive distress would lead to the discontinuation of experimental work and either termination of the animal or appropriate treatment under veterinary advice. Some treatments (e.g. stress exposure) can have extended effects on behaviour - although these are typically subtle and do not involve large changes in voluntary feeding/drinking or bodyweight. Excessive changes in behaviour or bodyweight loss would lead to the discontinuation of experimental work and either termination of the animal or appropriate treatment under veterinary advice.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

Rats - 1400 overall. Expected to be approximately 75% mild, 25% moderate.

Mice - 200 overall. Expected to be approximately 75% mild, 25% moderate.

What will happen to the animals at the end of the study?

- Kept alive
- Killed



Replacement

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

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

There are two main reasons why it is necessary to use animals to understand the psychological and biological mechanisms underpinning affective and cognitive processing.

The first is that affect and cognition fundamentally involve a behaving organism interacting with its environment. With respect to the investigations of disorders of affective and cognitive function, no currently available in vitro model systems can capture the full range of molecular, cellular, physiological and behavioural consequences of environmental or genetic risk factors for neuropsychiatric disorders.

The second is that animals are necessary for a range of other laboratory studies and the assessment of such manipulations on their welfare (and means to minimise adverse impacts) requires the use of the relevant species.

What was your strategy for searching for non-animal alternatives?

As noted above, the key questions for this project relate to the behaviour and biological function of whole animals. There are no non-animal alternatives for this project. That said, the animal based work will be supplemented by both ex-vivo analyses (e.g. of brain tissue) and computational modelling.

Why were they not suitable?

As noted above, the key questions for this project relate to the behaviour and biological function of whole animals - non-animal alternatives do not meet this need.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 primary basis for estimation was the level of work currently funded and in progress, with a projection of future funding at similar levels. These experiments have been planned using power analyses (where sufficient information exists to estimate effect sizes) or on the basis of Bayesian methods for cumulative data collection.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

I teach experimental design and statistics - as such, professional development includes keeping up-to-date with optimal design and analysis strategies. This knowledge will be applied in the design phases of all experiments to minimise animal numbers.



What other measures apart from good experimental design will you use to minimise numbers?

Where appropriate, pilot studies will be used to provide data for power analyses to determine optimal group sizes. Alternatively, cumulative data collection supported by Bayesian analysis will be used to ensure that only the minimal number of animals are used where power analyses are not likely to be accurate (i.e. in the absence of reliable information about effect sizes).

In addition, re-use of animals will reduce overall numbers. Tissue will be collected for post-mortem analysis - and shared where useful to others.

Finally, data will be archived for open access - supporting additional 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The animals used in this project will primarily be tested with reward-based procedures - the main scientific focus is on the processing of rewarding stimuli and the failures of this processing in models of risk factors for psychiatric diseases. Mild food or water restriction will be commonly applied to motivate consumption of rewards. However, the reward-based procedures are sometimes used in combination with - or comparison to - procedures that can cause pain or distress. For most applications of these aversive procedures, the experience of animals will involve mild procedures such as injections or exposure to short-term stressors. Individually, these cause only transient harm - but in combination or with repeated application they can contribute to cumulative suffering. A subset of the animals used in this project (approximately 12.5%) will receive the surgical implantation of an oral cannula under general anaesthesia.

These behavioural methods were chosen to reflect validated tests of the hedonic (e.g. like/dislike), motivational (e.g. subjective effort for acquiring reward), and cognitive (e.g. bias to or away from rewarding or punishing stimuli) processing of rewarding and punishing stimuli. Although some harms are inherent in these procedures (e.g. nausea from exposure to lithium-chloride), they have been designed to involve the minimum of harm consistent with the scientific purpose of the project.

The animals to be tested are chosen to isolate the effects of known risk factors for psychiatric disorders - including genetic and environmental risks (e.g. exposure to stress), or appropriate control animals to investigate normal functioning. Because the project is based largely on behavioural testing, models that produce severe harm or distress would be entirely inappropriate as this would interfere with voluntary behaviour.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?



Affect and cognition are properties of a whole, behaving, animal. They cannot be fully investigated under terminal anaesthesia. In addition, for the work to be directly translatable to humans, a mammalian species is also needed. The majority of the studies will use rats, because they have a high degree of behavioural flexibility and there are optimised behaviour-analysis methods appropriate to the scientific goals of the project for rats. In some cases mice will be used where genetic manipulations of interest are only available in mice.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Because the bulk of the experimental work on this project uses behaviour analysis, it involves regular (typically daily) contact with the experimental staff. Disruption of ongoing behaviour is a particularly sensitive sign of ill-health - often occurring before clinical observation could detect changes. Thus the experimental work for the project itself provides an additional layer of monitoring.

One of the aims for the project is the investigation of potential harms produced by common laboratory procedures (e.g. injections). If this work reveals unexpected harms from such procedures, they will be modified to reduce those harms (e.g. changes in the method of restraint for injections).

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

There are no specific best practice guidelines for the specific behavioural procedures used on this project (many of which have been developed/optimised for bespoke use in this laboratory). More general best practice guidelines (e.g. for husbandry or blood sampling) provided by the NC3Rs will be followed, as will the recommendations of the BUAAW/FRAME/RSPCA/UFAW working group on refinement of procedures for administration of substances.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The REDACTED has the benefit of an REDACTED and runs annual 3Rs workshops. I also subscribe to the NC3Rs newsletter. In addition, previous scientific work includes direct contributions to the 3Rs. Thus general academic reading includes coverage of the 3Rs as well as coverage of the behavioural techniques to be used on the project.

The behavioural methods to be used are already optimised for their scientific purposes, and chosen as the most well refined methods for 3Rs purposes on the basis of current knowledge. Should further refinements be made, they will be implemented as they have been in previous work undertaken on past projects (e.g. the use of lick analysis assays of hedonic behaviour to replace some taste reactivity tests previously based on the use of surgically-implanted oral cannula). In addition, 3Rs advances in husbandry (e.g. tunnel rather than tail handling of animals) will be adopted when they are demonstrated (e.g. work on a previous project included a contribution to the demonstration of the general improvements in mouse welfare produced by tunnel handling).

Explain the choice of species and the related life stages



There are two main reasons why it is necessary to use animals to understand the psychological and biological mechanisms underpinning affective and cognitive processing. The first is that affect and cognition fundamentally involve a behaving organism interacting with its environment. With respect to the investigations of disorders of affective and cognitive function, no currently available in vitro model systems can capture the full range of molecular, cellular, physiological and behavioural consequences of environmental or genetic risk factors for neuropsychiatric disorders.

The second is that animals are necessary for a range of other laboratory studies and the assessment of such manipulations on their welfare (and means to minimise adverse impacts) requires the use of the relevant species.

Rats and mice are used because they are mammalian species that afford translation to humans, allow the implementation of targeted genetic manipulations, and display high levels of behavioural flexibility needed for the experimental procedures to be implemented.

Adult and juvenile animals will be used to reflect both stages of development and the fact that early-life experience can impact on adult behaviour and function.





2. Age and agents to minimise radiation-induced cardiovascular disease

Project duration

5 years 0 months

Project purpose

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

Key words

Age, Ionising radiation, Atherosclerosis, Cardiovascular disease

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Cardiovascular disease is one of the most common cause of death in developed world. The main cause of cardiovascular disease is atherosclerosis, which is a swelling inside the wall of blood vessels (plaque) causing obstruction to blood flow that may result in heart attack or stroke.

Exposure to radiation is known to increase the risk of cardiovascular disease development and there is some evidence that age at exposure may affect this risk. However, this has been shown in cultured cells and it is unknown if a whole animal would respond in the same way. There are also drugs that we have shown to reduce early stage development of atherosclerosis in cultured cells, but again it is not known if these effects will be seen in the complexity of a whole organism.

We will investigate in a systematic manner to meet our objectives:

Establish whether age at exposure to ionising radiation affects likelihood of cardiovascular disease development.

Discover whether drugs can reduce the development of atherosclerosis following radiation exposure.



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

What are the potential benefits that will derive from this project?

Establishing whether age at exposure to ionising radiation affects likelihood of cardiovascular disease development would allow for individual risk assessments and practical use of the information. For example, those that have a higher chance of developing radiation-induced cardiovascular disease can have the amount of radiation they receive kept to an absolute minimum during medical and occupational exposures. Whereas individuals from an age group that is less sensitive may be able to tolerate higher doses without additional risk of cardiovascular disease; therefore, beneficial medical/economic effects of additional scans, radiotherapy treatment for cancer or time spent in areas with increased levels of radiation can be achieved.

In addition, the project will test drugs that have been shown to reduce the number of senescent cells (cells that no longer divide but secrete harmful substances) that have been shown to be central for atherosclerosis development. These drugs are already licenced to be used in humans for other purposes and could be re-purposed to minimise cardiovascular disease.

Both aspects of the project will also help scientists to understand the links between person age, cell age and radiation-induced cardiovascular disease. Long-term this will be the first step in the development of new approaches to reduce radiation-induced cardiovascular disease in the population.

The primary aim of this project is protection of human health, however there may also be veterinary application.

Species and numbers of animals expected to be used

- This project will use a maximum of 530 mice over 5 years.

What types and approximate numbers of animals will you use over the course of this project?

Initially 24 mice will be used in a pilot experiment to establish the most suitable X-ray dose to study atherosclerotic plaque formation in a mouse model that has not been used for radiation research before and so there is no existing data available.

Then we will use up to 200 mice in the first part of the project to establish the effect of age-at-exposure on radiation induced atherosclerosis.

If these results are clear, we expect to use up to a further 240 mice in phase two of the project to treat with drugs following radiation exposure; with the aim of preventing radiation-induced cardiovascular disease. If a significant age effect is not seen in stage one, only 120 additional mice will be required, as mice of a single age group only will be tested.

All mice used will be genetically altered strains that lack a gene involved in controlling cholesterol levels (Apoe^{-/-} or Ldlr^{-/-}) so are predisposed to form atherosclerotic plaques.



These mouse models have been proven as a successful model in previous atherosclerosis research.

Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The project is anticipated to have no adverse health effects. It is possible that a small number of mice may experience moderate effects as a result of exposure to ionising radiation or the effects of the genetic background of the model and associated atherosclerosis development, although this is unlikely within the timeframes proposed.

The animals will receive an X-ray dose (0.5-4 Gy, depending on strain) at either a young or older age, which may result in a small and transient weight reduction. This is most likely to occur in the pilot experiment where the response to radiation has not been tested previously. Any mice receiving drugs are expected to have a beneficial effect on their health. All mice will be monitored daily, those showing any adverse effects will be monitored more closely and any signs of severe pain, suffering or will be euthanised. Mice will be kept for up to 6 months following X-rays (or sham) exposure to allow atherosclerotic plaques to develop.

The mice will be group housed and provided with enrichment. At the end of the experiment all animals be killed by a humane method, after which, tissues (blood, aorta and other organs) will be removed for analysis to assess atherosclerosis development and ageing in the mice.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

We have set up and tested a method to investigate atherosclerosis resulting from radiation in cultured cells isolated from humans, including young and old donors to determine age effects in this non-animal alternative model. Having established the mechanism of the early steps involved in radiation-induced plaque formation and assessed the effect of drugs in this system, it is necessary to confirm these results in an animal model.

Lower species such as non-vertebrates and fish are not appropriate for this research as they have very different circulatory systems and have not been proven to be a good model for studying atherosclerosis.

Numerous resources including NC3Rs, PREPARE guidelines and Norecopa have been used to investigate additional non-animal alternatives.

Reduction

Explain how you will assure the use of minimum numbers of animals.



Good experimental design (including randomising treatments and blinding) have been used to maximise effect and reduce unwanted variability. We have also used all existing data available from our own cell culture models and other researchers that have used these mouse models to gain a best estimate of the expected effects. Therefore, group sizes and overall number of mice used can be kept to a minimum while still getting a clear result on the effect of age on radiation-induced atherosclerosis and the use of drugs to prevent this.

For the ApoE^{-/-} mouse model, we are using a low dose of radiation, but slightly higher than the minimal dose published to have an effect. This is to provide higher power of the data while using fewer animals (without significant increase in negative effects). As radiation effect in LDLr^{-/-} mice has been studied, the inclusion of a small pilot will prevent using larger numbers in the main experiment with uncertain outcomes.

Throughout the project, we will consider results that may allow a reduction of mice used in later stages. For example, if our pilot experiment shows that LDLr^{-/-} mice do not demonstrate radiation-induced atherosclerosis then no further experiments will be carried out in this strain, considerably reducing overall numbers.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The removal of tissue for analysis only after the death of the animal means procedures in living animals are reduced. Post-mortem techniques required will also be practiced beforehand on cadavers to ensure they are carried out correctly on experimental animals.

Drugs will be used at doses reported not to be toxic to the animals and where possible will be provided orally in food/water to minimise handling and distress.

Genetically altered mouse models will be used in order to study plaque formation, which does not occur in normal mice fed a normal diet, and also prevents feeding a high-fat diet that can allow plaque formation but results in obesity and metabolic syndrome.

A single sub-lethal X-ray dose will be selected that is the minimum dose required to see a clear increase in plaque formation.

We have chosen a radiation dose and end-point where we anticipate that plaque development will have progressed enough for analysis, but before any negative effect is expected to be experienced by the mouse.

All animals will be provided with comfortable group housing and enrichment. We do not anticipate the animals to experience any pain, but analgesics and increased monitoring will be used if considered appropriate.



3. Analysis of Fish Development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Zebra fish	embryo, neonate, juvenile, adult, pregnant
Giant Danio	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 is the aim of this project?

We use fish to investigate how, from a single fertilised egg, cells organise to form organs such as the eye, or how nerve cells connect to form the central nervous system. We would like to understand how the brain receives information from its environment, and, ultimately, how it processes this information to evoke behaviour 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?

The projects described in this application will contribute to a better understanding of a range of human eye diseases, as well as human brain diseases and their effects on behaviour (e.g. autism, dyslexia, schizophrenia), and thus contribute research data that can be used in drug development, or for diagnostics in the health system and, thus, will ultimately benefit human health. In addition, we are using the fish to understand defects in cell functions that lead to diseases linked to abnormal levels of metals in the cells. Such changes can cause, for example, childhood Parkinson's disease and, in collaboration with others, we are trying to find new drugs for treatment.



Our proposed programme of research will contribute knowledge of genes that are candidates for inherited human eye (ocular) diseases, a better understanding of the processes that these genes control, and novel animal models of human eye disease phenotypes. As such, our research is of relevance to and has the potential to have impact upon a) clinical research scientists, who will use our data to screen cohorts of patients for genetic changes (mutations); b) pharmaceutical industry researchers who could develop our novel disease models and transgenic animals for drug screens. Our knowledge will provide the basis for understanding the genetic and mechanistic basis of eye disease phenotypes such as microphthalmia (small eyes), anophthalmia (absence of eyes) and coloboma (the failure to close the eye cup completely). The information that we acquire from genetic analyses of zebrafish with eye defects will be shared with collaborators who will screen for comparable genetic defects in human patients with eye malformations. Our studies exploit novel techniques and resources to bring an unprecedented level of insight to the complex events that characterise early eye development and through this will help us to diagnose, model and understand human eye disease phenotypes. Proteins we study in the eye are also involved in the growth of other tissues and organs and some have been linked to cancer. In parallel, we will continue to investigate the mechanisms underlying the specification, function, behaviour and survival of specific classes of stem cell in the eye and brain which will allow us insight into which processes are affected in certain developmental defects (such as Human coloboma; 1:20,000 live births) or human degenerative diseases affecting the eye and the brain, as well as understanding signals that influence cell divisions in these cells. Thus, our research will benefit understanding of cancers in the nervous system, with their key hallmark of uncontrolled cell division.

Our studies of the mechanisms of differentiation of neural (brain) tissue into different brain nuclei and their connections (brain circuits) has led us to address links between defined brain circuitry and animal behaviour. Our current area of specific interest and expertise lies in asymmetry of the central nervous system (CNS), the brain. Altered CNS asymmetries are thought to be involved in a variety of human disorders from schizophrenia, dyslexia, depression and other mood disorders, as well as neuro- degenerative diseases such as Alzheimer's disease. Diagnosis of these disorders is often problematic and thus the number of officially diagnosed persons is likely to be a low estimate. However, it is estimated that more than 1 in 100 people may be affected by autism spectrum disorder in the UK (about 700,000), a similar number of people suffer from schizophrenia. Some people suffer a schizophrenia episode at least once in their lifetime and for some it becomes a chronic disease. Treatment is often restricted to pharmacological intervention; a better clinical outcome is achieved when treatment commences at a pre-symptomatic stage. Zebrafish show a range of complex behaviours from 5 days post fertilisation and offer a valid alternative to using rodents (mice or rats) for preliminary behaviour analyses and to test which small molecules, such as drugs, are effective to bring about a change in behaviour. For our research we are using and developing new behaviour tests for the zebrafish to make models for diseases we see in humans in which behaviours and brain function are affected. We are using these tests together with trying to identify new drug targets and find new genes that lead to disease symptoms. We hope that we will identify biological markers and molecules that may lead to the identification of drugs that are more effective for treatment in humans as well as help with early genetic diagnosis.

Finally, we are using zebrafish to model some aspects of early childhood diseases that affect metal metabolism in tissues and cells. In humans, changes in the concentration of metals, such as manganese for instance, leads to early onset Parkinson's disease affecting movement and cognitive functions. One therapeutic route currently tried in humans, is the use of drugs (chelators) which bind to metal ions in the cell and thus lower



the effective cellular concentration. However, there are currently no drugs that specifically target, for example, manganese metabolism, thus patients treated with such 'chelators' have less severe Parkinson's disease presentation but suffer from side-effects such as osteoporosis. Therefore, developing and testing drugs that target the metal transporter defect more specifically is vitally important. Our research not only facilitates correct diagnosis of patients with such diseases, but may also provide an avenue for effective treatment.

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

We will have identified new candidate genes that are likely required for normal eye development. We will also have progressed in our understanding of the genetic interactions of different genes and what genetic conditions predisposes a person to suffer from the eye diseases we study. This information can be compared to current data bases of information of patients with abnormal eyes and vision defects. In addition, there has been a big effort to sequence all the genes of 100,000 (and more in the future) people with rare genetic diseases. We are collaborating on this project and we will use information from the project as well submitting information gained from our work.

We will have a greater understanding of the processes and proteins that maintain cells as more stem cell like progenitor cells. We are studying the properties of this important class of cells, stem cells, in the eye and also in the brain. What factors in their environment or within the stem cells helps them to maintain the stem-cell -like character and which factors push them towards committing to a specific cell fate, such as a specific class of eye cells or nerve cells?

We will have a better understanding of the involvement of specific neurotransmitters in the brain of fish that are involved in specific tasks, for example exploring their environment. We will compare normal (wildtype) fish with fish in which we have changed the function of genes which might be defective in human diseases and mood disorders. This will give us a handle on how a fish reacts to its environment, for example with increased anxiety. We will build an 'atlas' showing where and when different molecules are released during brain activity. This can be used by other researchers and avoids duplication of work

We will have tested new molecules in our attempt to identify those that are more effective in helping with the symptoms of childhood Parkinson's disease.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Several human genetically inherited eye diseases arise from abnormal cell specification or cell behaviour at early stages of eye development. Our proposed programme of research into the development of the eye in health and disease will contribute knowledge of genes that are candidates for human inherited eye diseases, a better understanding of the processes that these genes control, and new animal models of abnormal human eye phenotypes. As such, our research is of relevance to, and has the potential to have impact upon a) clinical research scientists, who will use our data to screen groups of patients for mutations; b) pharmaceutical industry researchers who could develop our novel disease models and transgenic animals for drug screens.

In parallel, we will investigate the mechanisms underlying the specification, function and survival of stem cell in the eye and brain which will allow us insight into which processes



are affected in certain developmental defects or degenerative diseases affecting the eye. Our research data on stem cell maintenance and survival will give important information for potential clinical use in stem cell treatment/replacement in degenerative diseases of the eye tissue (for example due to damage, genetic predisposition or age) or for degenerative diseases affecting specific areas of the brain.

Our study of neuronal circuit formation aims to link functions of neuronal subsets to animal behaviour. Our area of specific interest and expertise lies in brain asymmetry. Altered brain asymmetries are implicated in a variety of human disorders from schizophrenia, dyslexia, depression and other mood disorders, as well as neuro-degenerative diseases such as Alzheimer's disease. The zebrafish offers a valid alternative to using rodents for preliminary behavioural analyses and to test the efficacy of novel drugs. The zebrafish community (and other research communities) benefits from the atlas showing whole brain activity.

Besides informing other researchers and clinicians, we hope that patients are benefitting from novel drugs more specifically targeting problems with metal transport into and out of cells and help with symptoms of movement and learning disabilities. Our research not only facilitates correct diagnosis of patients with this disease, but may also provide an avenue for effective treatment.

How will you maximise the outputs of your work?

In addition to publishing our findings in scientific journals, we present our work online on our website where anyone can download information and read about ongoing projects. Our work proposed in the application will generate a wealth of data on gene interactions in the eye that are of interest to developmental biology as well human genetics communities and we will create a data base with the information from our genetics screens that is readily accessible. Our data on the functioning of brain networks during specific tasks and genes that are necessary to build and maintain the left-right difference in our brain will be shared with other researchers and our project of the brain atlas will be a reference tool for the whole scientific community and will be made available online.

We also present our research as invited speakers at conferences or research institutes which allows direct exchange of information, including the best (or worst) approach to a particular scientific question. Finally, having worked in my chosen field of research for over 30 years, we have a vast network of collaborations with individuals as well as institutes in the UK and all over the world. We often have visiting scientists working with us to learn new technologies, similarly, we send our researchers to other groups to broaden their technical skills. We also have direct collaborations with clinicians working on eye diseases and also metabolic diseases in children.

Species and numbers of animals expected to be used

- Zebra fish: 222,000
- Other fish: No answer provided

Predicted harms

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



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The vast majority of the fish we use (>90%) will be for maintaining the fish stock and for breeding to provide us with eggs to use in our research. A smaller number will be used e.g. for studying animal behaviour where we watch their response to another fish, or another stimulus. In some studies we might add substances to the water to study the effect of a drug on the animal's behaviour, or we might look for a drug that will treat a disease or abnormal behaviour.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We do not expect that most animals will be much affected. Most animals are used for breeding and in behaviour analysis, and, generally, the animals should not experience pain. Experimental animals might experience temporarily mild discomfort or mild pain. On rare occasions, animals might experience moderate discomfort or longer-lasting mild discomfort or mild pain. We do not expect weight loss, tumours or other gross abnormalities. The possible exception are animals which might, for example, experience some effect on their movement (swimming) behaviour in our model of human diseases that affect the nervous system.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

Most of our fish (about 88%) should experience levels of pain and discomfort that is less than mild pain and discomfort, and should not affect them. Some fish (about 10%) might experience mild discomfort, not more than the prick of a needle. In a small number of animals (<5%) it is possible, although unlikely, that there is a temporary moderate discomfort. All animals will ultimately be killed using humane euthanasia methods in accordance with guidelines.

What will happen to the animals at the end of the study?

- Used in other projects
- Kept alive

Replacement

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

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

Our studies examine complex interactions between the cells in the organism and their organisation into tissues and organs in the developing embryo. In particular, we focus on how the eye develops from the embryo to the adult animal and how information that is received by the sense organs is processed by the brain. Finally, we study how information received by the animal via their sense organs and the organisation of the brain influence how an animal behaves.



The eye of a vertebrate such as a fish or human, the complexity of the brain organisation, and animal behaviour such as social preference, are very different than the eye, brain and behaviour we see in less sentient animals such as worms or flies. We are also trying to model human diseases of the eye and nervous system which we cannot study in less complex animals, or in a tissue culture dish or in a computer simulation.

Zebrafish is an ideal model because its brain is a simpler version of that found in mammals such as humans. By studying zebrafish, we are using an animal considered less conscious/aware than mammals to achieve our research goals and to answer our scientific questions.

We have, however, started a pilot project using the nematode *C. elegans* for some of our genetic analysis into the genetic and molecular interactions of proteins thought to be required for normal eye development, as well as identifying novel players. This represents a true achievement of replacement.

What was your strategy for searching for non-animal alternatives?

We use bioinformatics and computational analysis to analyse our data from animal research in the most efficient way.

We are using animal research data to build a standardised reference brain atlas to give temporal and spatial information of the expression of neurotransmitters and other small molecules in a behaving animal, that is an animal involved in a behavioural response.

We have developed an 'organoid' system of the developing eye in which we can study the less complex cellular interactions of early eye development. This system, although useful, has its limitations as environmental factors from surrounding tissues, including tissue tension, cannot be modelled in this system yet. The 'organoid' eye is radially symmetrical, unlike the normal eye.

Why were they not suitable?

The vertebrate eye is a very complex structure. We cannot study the role that particular proteins play in the development of a normal eye, or what happens when they do not function properly except in the whole animal. Similarly, we are studying how the left and right side of the brain become different in their structure and function during development, similar to that what is seen in humans. This cannot be modelled adequately on a computer. Lastly, we study the complex interaction of an animal with its environment, again this has to be done using animals and modelling can only give us suggestions of what to explore further.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 fish are estimated based on our experience on how many fish we need for breeding, and on power calculations for animals used in experiments. The majority (ca 75%) will be used for the generation of new genetic lines and for breeding and maintenance, about 60,000 (ca 25%) will be used to study, e.g. animal behaviour.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We use statistical design to determine the number of animals we need to use for each experiment to get a valid result. It is important to balance the need to have statistically significant results with the desire to reduce the number of animals we use as much as possible.

We are also building a standardised reference brain which, once established, will reduce the number of animals that are used dramatically. REDACTED

We are also using the nematode *C elegans* for some pilot studies of genetic interactions, instead of fish.

What other measures apart from good experimental design will you use to minimise numbers?

Most fish we use are for the generation of new fish lines. We have developed new techniques that allow us sample fish that might be of scientific interest to us at the embryo stage instead of having to wait until they have reached sexual maturity to identify those of interest.

We also will be using worms instead of fish for some preliminary studies to identify new interactions of proteins involved in the normal development of the eye.

Finally, we use bioinformatics, i.e. using computers to analyse our data to ensure we make excellent use of all data we received from our experiments. This is especially helpful in finding genetic changes that might be the causes of diseases or developmental abnormalities. This also helps to share our information with the zebrafish community locally and internationally and avoids duplication.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We are using the zebrafish to model the development of the eye and the brain in health and in disease. Unlike mammals, zebrafish lay their eggs externally and we can simply collect them from their home tank, no harm done to the females laying the eggs. The embryos are transparent which allows us to gain much information by simply examining them under a microscope.



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

A large amount of our work is using animals at the immature, embryonic stage or after after they were killed humanely. Only for some of our studies we need to use larvae (young fish). For example, in our study of animal behaviour, we need to use larvae that show a robust swimming behaviour, and in some cases are old enough to show social preference. We are using the least sentient model animal that shows a similar eye and brain structure and organisation as we see in humans.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We are making every effort to balance harm/benefit and to refine our methods and to use fewer animals in accordance with the 3Rs.

Advances in 'reverse' genetics now enable us to generate mutations in specific genes or establish transgenes at specific places in the genome. Thus, for genes of interest, we can now induce targeted mutations as well as test for 'rescue' of a defect instead of generating a large number of randomly mutagenised fish to be screened for a specific mutation. This results in a substantial reduction in the number of animals that are used to identify a specific mutation. In addition, the transgenic lines generated are incredibly useful to answer our research question more specifically and often non-invasively, and thus helping to reduce the number of animals that have to be used to obtain valid data, as well as severity levels, in observance of the two of the 3 R's, Reduction and Refinement.

If a new effective method to identify transgenic and mutant animals in embryos or fry is established that avoids fish to be grown to maturity, e.g. by removing small number of cells in early embryos to be used for genotyping instead of fin biopsies, we will adopt a different method for identifying genetically altered animals.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Local AWERB guidelines; ARRIVE guidelines of the NC3Rs; guidance on Animal Testing and Research from the Home Office; Good research practice guidelines from the Wellcome Trust; LASA and RSPCA guidelines; PREPARE guidelines.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

For example, the information given on the websites of organisations involved setting policy and also in the advancement of the 3Rs (e.g. the National Centre for the Replacement, Refinement and Reduction of Animals in Research; Institutional guidelines, Home Office). In addition, I stay informed through communication with my project manager who is a longstanding member of our institute's Animal Welfare and Ethical Review Board. In conjunction with other longtime senior members of my group, we ensure implementation of the 3Rs and Home office guidelines, and monitor compliance and training of all members of our research group.

Explain the choice of species and the related life stages



Our studies examine complex interactions between cells and how they become organised into tissues in the developing embryo. In particular, we focus on the interactions between structures of the sense organs and the brain, and how an animal reacts to signals from its environment. We cannot model the development of the vertebrate eye and brain adequately in animals such as worms or flies or in a laboratory dish. Wherever possible, we are, however, also using computational analysis of our data to help us reduce the number of animals we use in our research. We have also started to complement our work in the fish with preliminary research on how specific gene and their proteins, that are candidate genes for eye diseases, interact in the worm *C. elegans*.

Zebrafish is an ideal model because the way its brain and eye are build is a simplified version of that found in mammals such as humans. By studying zebrafish, we are using an animal considered less aware than mammals to achieve our research goals and to answer our scientific questions. Through our work we have contributed to reducing research with mammalian species.

For most of our studies we will use very young embryos without a mature nervous system. However, to study how an animal behaves in response to its environment we will need to use young fish that can swim and show social behaviour.





4. Analysis of psychological stressors in cancer

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

The aim of the project is to understand how stress can influence how cancer develops, progresses and spreads in mice. In addition, we aim to test novel treatments such as stress hormone blockers and immune modulators on improving cancer progression and response to treatments. The project is focused on 3 cancers; breast, ovarian and prostate because these cancers are all solid tumours, and also because in some cases they have a gene in the gene called BRCA1 which can make these cancers aggressive.

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

Why is it important to undertake this work?

1 in 7 women will develop breast cancer, 1 in 8 men will develop prostate cancer and ovarian cancer is one of the deadliest diseases. It is well established that stress (e.g social isolation, anxiety and depression) can play a role in cancer progression; however much of this work is based on epidemiological evidence and the exact mechanisms are currently still not yet known. In addition how stress affects treatments are not fully established. The immune system, which is also affected by stress hormones, plays important activating and suppressing roles in controlling cancer. Tumour cells can evade immune recognition. However, the cellular mechanisms whereby stress hormone exposure mediates changes



in immune cell trafficking, function and the effects of stress on the outcome of immune therapies are largely unknown.

Therefore this project will result in a better understanding of the link between stressors, the immune system and disease progression to improve treatment strategies and patient outcome.

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

1) Understand the effects of psychological stressors on cancer progression and response to drug treatment in mice bearing human xenografts of breast and ovarian cancer and syngeneic mouse models.

New information:

Understand how different types of stress can impact the immune system and cancer spread

Determine if stressors can impact cancer progression by measurement of tumours.

Determine if stressors can affect the response to drug treatments such as chemotherapy and PARP inhibitors.

2) Analyse the role of the REDACTED in cancer

New Information:

i) Determine the Impact of stress and the microbiome on tumour establishment and spread

3) Use of stress and immune therapies and novel drug delivery methods as a strategy to treat cancer using the models described above.

Our long-term goal is to identify new personalised approaches to improve outcomes for cancer.

1) test the central hypothesis that stress influences cancer progression by modulating immune cell recruitment into the tumour and how it works e.g killing of tumours.

ii) test combined treatment of immune therapies with a stress hormone receptor blocker (propranolol or Mifepristone and other glucocorticoid receptor blockers) to improve treatments of cancer.

These will lead to publications in high quality journals such as breast cancer research, cancer research etc.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

What are the benefits?

This project will allow us to develop a better understanding of the signalling mechanisms involved in stress, the immune system and cancer and further help us understand the role



of the REDACTED in treatment efficacy. Ultimately, the results of this research will allow the improved diagnostics and drug efficacy in cancer

Who will benefit

Patients with breast, ovarian, prostate cancer, clinicians and academic researchers will benefit.

How they will benefit

To improve benefactor's understanding of 1) stress management; a major societal impact, 2) improve efficacy of drug treatments and design of clinical trials; resulting in a refined management of economic resources 3) Improve our understanding of stress in the context of disease and use novel technologies to treat and diagnose disease.

When will they benefit

The short term benefit will be that our findings are published in peer reviewed journals within 1-2 years (please see results from last project license to prove this). The longer term benefit is that we aim to translate this animal research to a clinical trial and interventions within 5-10 years. For example, Researchers in MD Andersen, USA discovered that catecholamines can influence ovarian cancer progression and within 5 years clinical trials are being designed using beta blockers to limit catecholamine influence.

How will you maximise the outputs of your work?

Scientific: Data from this proposal will be published in high impact journals such as Cancer Research, and will be presented at both national and international conferences (e.g. American Association for Cancer Research, National Institute of Cancer Research Annual meeting).

REDACTED We plan to promote and discuss our work at patient forums by giving talks and offering leaflets on our research. We will hold forums such as public lectures and Q & A sessions and also to promote a road show stall in a charity event such as Independent Cancer Patients' Voices. Also, the PI and team plan to engage in the electric 5 k run for cancer charities to raise funds for the charity and wear T shirts to promote their work. I will offer lab tours"-about 4x/yr for their fundraisers and volunteers - lay members of the public

Species and numbers of animals expected to be used

- Mice: 1000 BALB/c and 1200 C57BL/6

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Stressors



To assess the effects of psychological stress on immunity and cancer progression, groups of mice will undergo restraint stress: standard confinement in a tube that prevents turning, or social isolation: single housing in a standard cage. After stress, mice will be killed humanely and sera will be collected immediately following restraint stress.

Immune Adoptive transfer

Mice will be injected with cells into the dorsal flank. Some groups will receive immune cells adoptively transferred from mice above.

Cancer cell injections

Mice will be injected with ovarian and prostate cells by IP injection on week 1 and by injections into the mammary fatpad for breast cancer cells.

Stress hormone blockade.

For cortisol blockage, glucocorticoid blockers will be administered daily by oral gavage or by IP injections. Mice may receive drugs through an Alzet minipump.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Mice will undergo tumour implantation and growth of tumour to a maximum size of 1.2 cm (1.5 cm for treatment experiments). This will result in some mild to moderate discomfort. Mice will receive treatments by injection resulting in mild and transient pain and discomfort. Some mice will be exposed to a stressful situation: restraint for 2 hours per day or singly housed for 4 weeks.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

Moderate (100%).

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Investigations using these models will allow cellular mechanisms of cancer to be studied in detail as well as the effectiveness of novel diagnostics and therapies. These animal models of cancer must ultimately be used as these are the models which most closely resemble the pathophysiology observed in patients. More specifically, these models are able to reproduce the complex interactions observed between different systems which prevail in cancer such as the interactions between the immune and nervous systems.



Many years of research mean that we now have established animal models of stress and cancer.

What was your strategy for searching for non-animal alternatives?

In-vitro work such as cell line models are considered to be a replacement alternative, and we are currently using in-vitro models of cancer in our lab. In addition we will be using human cancer tissue to develop organoid models of cancer to complement this research.

Why were they not suitable?

At present, cell culture methods cannot fully recapitulate the 3D nature of tissues, the diverse environment of differentiated tissues, or the bioactive compounds e.g enzymes found in the tissue and blood. In addition, cell culture methods necessarily use immortalised cells, which are cancerous in nature, rather than primary cells. Tissue culture cells are known to have different responses to the environment than primary cells, therefore cannot create a true environment for studying cell communication.

Reduction

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

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

We have used statistical methods to determine that we will require around 10 mice per experimental group and typically experiments will compare 3 or 4 groups. We aim to test around 5 different potential treatments in three types of cancer over five years.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We have used both expertise from statisticians and also we have used the NC3Rs' Experimental Design Assistant

What other measures apart from good experimental design will you use to minimise numbers?

From our current project license we have garnered enough information to allow to perform power calculations to reduce our animal numbers. In addition we routinely share tissue with several other PIs and these have included: brain, spleens, lymph nodes, gut tissue.

Refinement

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



Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use mice with or without a particular genetic changes because we can directly look at the effects of stress on cancer cells in the absence of a an immune system. We have carefully considered each of the procedures described in this application in order to minimise pain and distress experienced by animals, and to enhance their well-being. Anaesthesia and analgesia are used whenever appropriate and possible. Careful consideration has been given to the endpoints and measurable parameters obtained from each procedure and as much information as possible is gained from each animal. At the end of each experiment, the most humane method of euthanasia is always chosen.

We will inject the minimum number of cancer cells in the smallest volume into the mammary fatpad. Measurement variations will be minimised by ensuring that the same well-trained technician is involved for the duration of the study. For an animal carrying a single tumour, the mean diameter will not normally exceed 1.2 cm in mice.

Stress Method: We will use a 1h (acute) or 2 h daily over several weeks (chronic) restraint stress or social isolation.

Rationale: This is a well-established method for activation of the hypothalamic adreno pituitary axis resulting in elevations of stress hormones and to study the effects of psychological stress in rodents. We will use this method to examine the effects of stress on ageing, cancer spread and on the immune system.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The experiments proposed will use the simplest possible animal system, which has an intact immune and endocrine (stress response pathways) which is the mouse model.

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We weigh the mice weekly and if a procedure is more complex e.g insertion of Alzet pumps will monitor the animals daily and we have created a Standard Operating Procedure (SOP) for surgery in mice.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Before the start of any animal experiment we discuss this with our NACWO and write up a project outline. We also have SOPs in place for more complex experiments e.g. those requiring anaesthesia and surgery. We will follow the guidelines of Workman et al (2010) paper 'Guidelines for the welfare and use of animals in cancer research' British Journal of Cancer (2010) 102, 1555–1577 for tumour injections and the LASA Guidelines on administration of substances, LASA Guidelines on aseptic technique.

How will you ensure you continue to use the most refined methods during the lifetime of this project?



I will check the following website: <https://www.nc3rs.org.uk/> monthly for updates and will address any updates at our weekly lab meeting and disseminate this information to all personal license holders. In addition our NACWO and NIO often provides NC3R updates.

Explain the choice of species and the related life stages

The adult mouse model is relevant to our studies because they are the lowest species in which we can address our questions regarding stress, immune and cancer models. The mouse model is most widely used and accepted animal system for these type of studies





5. Animal Models of Human Disease

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Animal types	Life stages
Rats	juvenile, adult, neonate
Mice	juvenile, adult, neonate
Guinea pigs	adult, juvenile

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The objective of this project licence is determine how the body processes potential new medicines and whether these new medicines are a cure for human diseases.

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

Why is it important to undertake this work?

It is important to perform this work because there are still many human diseases (e.g. Alzheimer's, lung fibrosis) for which there are no cures or because the cures that exist are not effective.

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

The outputs from this project licence will include the following;



Study reports which describe how the body reacts to the new medicine and whether the new medicine is likely to be effective if given to human patients.

Peer-reviewed publications

Data presentations at conferences/internal meetings Blogs/webinars (internal and external)

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Enabling the development of successful medicines will benefit society through diagnosis, treatment or prevention of disease.

Identification of toxic reactions can prevent future harms to human volunteers or patients by resulting changes to drug development programs.

The wider scientific community will benefit from publication of data demonstrating improvements in study conduct that improve the relevance of the animal model or lead to refinements and reductions in animal usage.

How will you maximise the outputs of your work?

In-house collaborations and information exchange with others within the organisation worldwide, identifying successful and unsuccessful approaches.

Collaboration with clients (knowledge gained on products).

Hosting scientists and animal carers from other establishments, including universities, to promote best practice.

Presenting outputs at international scientific conferences. The project licensee also liaises with local universities to share best practices.

Species and numbers of animals expected to be used

- Mice: 15,000
- Rats: 10,700
- Guinea pigs: 600

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Many of the studies conducted in this licence require that lung injury is induced which leads to inflammation and sometimes lung fibrosis. Animals will be administered with



different agents to cause lung injury on one or more occasions. The process of administering agents to the lungs will be performed under anaesthesia and therefore the animals will not feel any discomfort from the process.

Animals will be then be treated with potential cures given by a clinically relevant dose route e.g. by mouth, by infusion into the blood or by inhalation to the lungs. All these dose routes are expected to cause only momentary discomfort. For injections into the blood, the injection site will be alternated to reduce discomfort. Treatments may continue once or twice daily for 1 or 2 days (e.g. short term studies) or for one month (e.g. longer term studies investigating fibrosis).

Animals maybe monitored during development of the disease using methods to assess shortness of breath; in this process, animals are placed in a small chamber and measurements taken. The animals are not expected to feel much discomfort during this respiratory assessment since they are free to move in the chamber. Generally, when they are first placed in the chamber they are investigate their surroundings (10 to 15 mins) and then fall asleep. However, it may also be necessary to asses breathing using a system in which the animals are held in restraint tubes and may feel some temporary discomfort. To reduce discomfort, the animals will be acclimatised to the restraint tube and offered a treat after the procedure. On average this procedure takes approximately 30 mins and the may be performed once on a short term study or a couple of times on a longer term study.

Animals may also be transported to another facilities (and project licence) for whole body imaging assessments as used in human patients. The animals may feel some temporary discomfort moving from their home cages to a travelling box and during transit. However, to reduce any discomfort, the animals will be transferred with their cage mates. The animals will have access to food and water during transit. Transport will be performed for same day delivery and in temperature controlled vans.

Other study types on this licence will investigate the development of new cough medicines and in these studies animals will be induced to cough for periods up to 20 mins one one or a couple of occasions. Animals may feel discomfort during the coughing period (about 20 mins) but soon return to normal once the coughing has subsided. Animals may be induced to cough a couple of times over a 2 week period; in our experience, repeated coughing has no detrimental effects.

Other study types are designed to investigate how new medicines are processed by the body animals. In these study types the new medicine will be administered by a clinically relevant route of delivery (e.g oral) and blood samples taken to assess drug levels. As described above, animals may feel a momentary needle-stick pain for blood sampling,. Typically, these types of studies take place over a 24h period and the animal exhibits normal behavior for the duration of the study.

Finally, some studies may be undertaken to assess severe acute lung injury as occurs in patients who have had major trauma. In these studies, animals will be anaesthetised and put on artificial ventilation from the start of the study and will therefore not feel any pain or discomfort. At the end of all studies in this licence, animals will be humanely killed under terminal anaesthesia.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.



Previous experience has shown that most animals with lung injury lose their appetite leading to weight loss. Animals also develop shortness of breath as observed by an increased rate of breathing. These changes are generally seen during the first few days of a study and gradually decline in longer term studies. Animals are supported in this phase by being offered high energy food supplements REDACTED to encourage weight gain. Also, the animals are very closely monitored during the study and charts are generated on a daily basis to monitor individual animals and humanely kill animals who exhibit other signs. In longer term fibrosis studies, the animals recover and are indistinguishable from normal animals.

In studies designed to evaluate new cough medicines, animals may cough for a 20 min period and therefore the extent of discomfort is expected to be temporary.

In studies designed to investigate blood levels of a new medicine, the animals may feel mild discomfort during blood sampling but will otherwise act normally.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

Using data from Home Office returns the following is expected

Mild: 80%

Moderate: 20%

What will happen to the animals at the end of the study?

- Used in other projects

Replacement

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

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

While non-animal methods are useful to identify test item effects on individual cell types and on individual chemical pathways, they are currently not able to predict effects on whole body systems or to provide information on how a body reacts to a new medicine. Therefore, it is not currently possible to acquire all of the information on how the heart, brain and lungs may be affected by new drugs, without using animals. This information is essential, to protect human volunteers and patients. Although the studies conducted in this project license are not governed by regulatory authorities (e.g. MHRA) it is generally accepted by scientists that non-animal alternatives are not able to predict the dose in humans of a potential new medicine.

What was your strategy for searching for non-animal alternatives?

Our laboratories, are developing and validating new in vitro assays to assess toxicity using human cells. However, these in vitro systems currently do not mimic all the processes that happen in the body. Therefore, it not yet safe to predict the safe dose of a medicine based on the results from non-animal alternatives.



Why were they not suitable?

There currently remains general scientific agreement that to protect human volunteers and patients, non-animal alternatives do not, as yet, provide enough information to replace animal studies. For example, it is not yet possible to use non-animal alternatives to predict a safe dose for the first clinical trial studies in healthy volunteers.

Reduction

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

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

The estimates are based on analysis of use of animals in an existing licence authorising work for the same purpose, combined with anticipated upcoming studies.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Statisticians have provided advice on experimental design for all standard study designs within the project. This ensures that studies are correctly designed to meet the study objectives. Statisticians will be consulted on a case-by-case basis for any study that requires a non-standard design.

What other measures apart from good experimental design will you use to minimise numbers?

Pilot studies will be used to investigate the potential of new designs to improve outcomes. These could include evaluation of new ways to analyse the data, or additional tests to conduct, leading to improved data quality and translation to humans.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Rats, mice, guinea pigs.

We use the above species because they develop aspects of the human disease in relatively short-time frame (e.g. days to weeks) that can be improved by known cures (if available) (e.g. antibiotics for pneumonia, codeine for cough).



Individual animals are carefully monitored during the study in the form of daily observations. Charts are prepared for each individual animal so that welfare is closely monitored after treatments and the study design modified to reduce any harm (e.g lower dose of the new medicine).

Potential medicines are given to animals by common routes (e.g. in food, by mouth, by IV); all these methods are well established and common methods for the species to be used.

Volumes of potential medicines given to animals are in-line with published guidance on minimising discomfort, and/or are known to cause minimal discomfort based on extensive experience at the site.

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

Collaborations are currently on-going to determine whether improvements in disease assessment could be implemented to reduce the study time from weeks to days in some models.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The species used are selected based on known standards of outcome which will answer the scientific questions.

To enable comparisons with other data being generated in the same species as part of the safety assessment of potential new medicines.

The use of terminally anaesthetised animals would not model human disease and treatment (apart from the model of Adult Respiratory Distress Syndrome which is being performed in terminally anaesthetised animals).

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Monitoring of on-going procedures will be refined as any causes for concern are identified; for example, reduced dose of a challenge agent.

The surgery and anaesthesia/pain relief protocols will undergo continual assessment and refinement to improve outcomes.

Continue collaborations to improve *in vitro* assessment (to better predict safe doses to humans)

Continue collaborations to assess the effectiveness of a new cures in days rather than weeks and therefore reduce the length of studies and number of procedures per animal.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?



Following guidelines from relevant working group publications (e.g. An Official American Thoracic Society Workshop Report: Use of Animal Models for the Preclinical Assessment of Potential Therapies for Pulmonary Fibrosis, 2017).

Dose volume and blood volume limits agreed with the animal welfare and ethical review body are based on the 2001 publication of REDACTED: A good practice guide to the administration of substances and removal of blood, including routes and volumes.

document from 2010 on dose level selection for regulatory toxicology studies.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We are involved with other groups working in the same field, and have participated in analysis and publication of data in the scientific field to assess methods of conduct and outcomes. This establishment has been involved with various working groups of the National Centre for the Replacement, Refinement and Reduction of Animals in Research over many years.

Explain the choice of species and the related life stages

New medicines are approved for use in human patients by governmental bodies (e.g. Medicines & Healthcare products Regulatory Agency) who authorise them as safe for the treatment and effective cures. The animal types used in this licence are recognised by regulatory bodies as providing information to help select a safe cure for human diseases.





6. Autonomic control of cardiac function and rhythm

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
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 is the aim of this project?

The overall aim of this programme of work is to investigate how the nervous system affects key processes in heart function and to gain an understanding of the relationship between dysfunction of this system and lethal abnormal heart rhythms (ventricular arrhythmias) which can lead to Sudden Cardiac Death.

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

Why is it important to undertake this work?



This licence is essential to advance the understanding of why sudden cardiac death occurs and the role that the nervous system plays. The experiments are designed to identify key signalling pathways that will further scientific knowledge, which we hope will one day improve current therapies and develop new ones.

A new emphasis of this PPL is that data collected will be combined with mathematical modelling to improve and advance what is currently available which will incorporate up to date information on structure and physiology of nerves which plug into the heart.

The current work is essential to advance the understanding of the mechanisms underlying autonomic modulation of ventricular fibrillation (VF) and cardiac function.

The investigations may identify key signalling pathways that could lead to the development of new therapies for this lethal condition.

Data will be published in academic journals that will be of interest to cardiac- and neuro-physiologists and clinical personnel with an interest in cardiac disease and the wider scientific community.

This PPL will follow the same theme of the previous PPL to facilitate the continuation of the research approved on our BHF programme grant. Of course, new data and new findings are accumulated everyday which drives the direction of the ongoing research and thus this application. The remit of the work in this PPL will still address the theme 'Autonomic control of cardiac function and rhythm' and will focus on molecular, cellular and whole heart models to investigate arrhythmia and sudden cardiac death.

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

This licence is essential to advance the understanding of why sudden cardiac death occurs and the role that the nervous system plays. The experiments are designed to identify key signalling pathways that will further scientific knowledge, which we hope will one day improve current therapies and develop new ones.

A new emphasis of this PPL is that data collected will be combined with mathematical modelling to improve and advance what is currently available which will incorporate up to date information on structure and physiology of nerves which plug into the heart. We aim to gain a better understanding into cardiac nerve distribution and ion channel distributions and the subsequent regional effects on the heart.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Data produced throughout this project will be presented at national and international conferences and published in academic journals.

Short term: Gain an understanding of the mechanisms underlying the modulation of arrhythmia which will inform studies using the myocardial infarction (MI) models of heart failure and enable comparison with disease models. Studies in cardiac cells will help us to identify ion channels that are important in modulation of arrhythmia. This data will also be used to determine relevant mathematical models of heart function. Colleagues and collaborators will benefit from data that will inform their experiments and approaches.



Medium term: The mechanisms and pathways identified will enable us to test target specific blockers/activators with potential to aid development of therapies. All of the above can be investigated in MI models to gain understanding on how these mechanisms and pathways are modulated in disease states. This data will also be used to determine relevant mathematical models of heart function. Colleagues and collaborators will benefit from data that will inform their experiments and approaches.

Long term: Advance the understanding of the mechanisms underlying ventricular fibrillation (VF) and cardiac function. The investigations may identify key signalling pathways that could lead to improvements in current therapies and the development of new therapeutic modalities for this lethal condition. Data will be published in academic journals that will be of interest to cardiac- and neuro- physiologists and clinical personal with an interest in cardiac disease and the wider scientific community.

How will you maximise the outputs of your work?

We will maximise the outputs of this group through collaborations with other departments within the REDACTED and with other universities. We will disseminate new knowledge at national and international academic meetings and conferences and through publication of our findings in scientific journals.

Species and numbers of animals expected to be used

- Guinea pigs: 250
- Rabbits: 1000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Heart preparations will be prepared and removed during non-recovery anaesthesia for animals entering protocol 1. This will involve subcutaneous and intravenous injections, tracheotomy to expose of tissues of interest and isolation and resection of vasculature / muscle (optional i.e. for innervated heart preparation), all of which will occur under terminal anaesthesia. The heart and associated tissues will be removed immediately after intravenous overdose of anaesthetic. Animals in protocol 1 may also have optional blood sampling under anaesthesia.

Animals from protocol 2 will have general anaesthesia and surgical procedures performed to tie of a coronary artery (or not for Sham animals) to produce a myocardial infarct. This will involve subcutaneous and intravenous injections, optional blood sampling under anaesthesia, thoracotomy and exposure of tissue of interest, 1 or more coronary arteries will be ligated to cause an area of ischemia and infarct for a MI (Sham animals will not have a coronary tie) and administration of intrathoracic steroids. Surgical subcutaneous implantation of a telemetry device is also optional. Animals will be allowed to recover for up to 16 weeks, after which they will be prepared for terminal experiments which will involve subcutaneous and intravenous injections, optional blood sampling, tracheotomy to expose of tissues of interest and isolation and resection of vasculature / muscle (optional



i.e. for innervated heart preparation), all of which will occur under terminal anaesthesia. The heart and associated tissues will be removed immediately after intravenous overdose of anaesthetic. Animals under this protocol can have optional administration of dietary supplements in food or water, for example Beetroot juice.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

To develop the heart failure model we perform an open-chest surgical procedure that is rated at moderate severity. Premature death is the most significant adverse effect that we try to keep to a minimum through sound aseptic surgical techniques and rigorous post-op monitoring with rapid reactive treatments if symptoms develop. Rabbits do not commonly display symptoms of heart failure like humans, but the most common adverse harm is pain, which is controlled with good pain medication. At the end of procedure, animals are anaesthetised and the heart is removed for use in the laboratory for scientific 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 species)?

500 Rabbits - Moderate (50% of total rabbits)

500 Rabbits – Non-recovery (50% of total rabbits)

250 Guinea pigs – Non-recovery (100%)

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 investigate modulation of VF in humans due to its lethal nature. To get a better understanding of the mechanisms involved in the initiation and development of arrhythmia and SCD whole heart models are essential to understand the spread of electrical activity and thus the regional electrophysiology. Cellular experiments can then investigate deeper into the specific ion channels involved and their heterogenous distributions in the heart. Hence animal models are essential to advance our understanding of the mechanisms involved. This data will inform the development of relevant mathematical models which will hopefully allow for some replacement in the future.

Currently there are no non-animal alternatives and no appropriate mathematical models available.

What was your strategy for searching for non-animal alternatives?



Mathematical modelling has provided some useful tools to investigate some aspects of cardiac physiology.

Why were they not suitable?

Mathematical models are in development, incomplete and are currently not suitable; as they do not fully address complex cardiac rhythms owing to the lack of a comprehensive understanding of all the processes involved.

To note: we have a 5-year BHF programme grant that will use our data to incorporate into current mathematical models. Through continued development this will hopefully allow some 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?

Group size calculation is informed by expected differences in parameters based on our previous data (e.g. heart rate, action potential duration, ventricular fibrillation threshold) to provide adequate statistical power.

These are calculated based on the standard deviation (SD) of the data and estimated detectable change (DC) of the parameters studied. The least number of experiments required to demonstrate a scientific effect are used. Experiments are carefully designed and performed under tight control conditions and analysed in acceptable small cohorts, so statistical differences are analysed as work progresses and discovered early, so unnecessary additional experiments can be stopped.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We perform experiments in-vitro as confounding effects of circulating hormones/autonomic reflexes in in vivo setting would increase variance

This allows our experiments to be tightly controlled from a homogenous population of animals, so the smallest number is used to achieve the objectives

Statistical analysis is discussed with our departmental statistician when needed

What other measures apart from good experimental design will you use to minimise numbers?

Use of good laboratory practice (GLP)

Rigorous experimental data appraisal to ensure quality and low failure rates

Sharing of tissue e.g. sharing of muscle tissue with collaborators

Covering more than one objective for each animal



Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Protocol 1

Amongst small experimental animals, Rabbits have the most comparable cardiac electrophysiological and ion channel characteristics to humans. The physiology of the Guinea pig action potential is different to rabbits and is more influenced by the potassium current known as I_K s which plays an important role in the development of arrhythmias. This is the most refined model for the intended purpose of investigating of the autonomic nervous system (ANS) as it allows tightly control studies without confounding influences.

For isolated heart, single cell, molecular biology and immunohistochemistry experiments, the isolated heart is the most refined method for these intended purposes. The preparation is harvested during non-recoverable general anaesthesia following a sub-cutaneous injection of sedatives, which is the least painful injection route which has been refined from previous use of intramuscular routes. Previously, animals were ventilated with room air but we have since refined this method with use of an oxygen concentrator which helps to maintain the rabbits oxygen saturation levels. We now also use continuous propofol infusion as oppose to boluses to allow better control of anaesthesia.

Protocol 2

Inducing myocardial infarction (MI) has clear adverse effects on the animal. The procedure involves a thoracotomy and the induction of a myocardial injury, which results in pain and other complications such as ventricular failure, thromboemboli and death. Experience with this model indicates that the expected harm can be controlled and minimised with resultant pathophysiology that can be used with human relatable data.

Refinements related to the MI model

Use of rabbits 2.5-3kg to reduce mortality rate seen previously in lighter rabbits

Refining anaesthetic protocols to ensure calm induction for the animals

Anaesthetise vocal cords with local anaesthetic before endotracheal tube (ET) tube insertion, without using the stylet

Steroids used prior to closing thorax to reduce adhesions

reduction in mortality rate from <30% to <20% (with refinement and control of procedures, our more recent data over the past 2 years would support a mortality rate of 5-10% of which the majority occur under anaesthesia)

Use of effective analgesic regimen



Frequent monitoring including pain scoring

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Rabbits are preferred as the structure and physiology of the heart is more similar to humans, than rats or mice. We use guinea pigs because the physiology of the Guinea pig heart is different to rabbits and is more influenced by potassium ion channel currents (e.g. IKs) which plays an important role in the development of arrhythmias.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Use procedures designed to cause the least pain, suffering or lasting harm

Transport stress is minimised by acclimatising animals to handling and animals are box trained prior to delivery and assessed as fit to travel before transport.

Pre-emptive, intra- and post-operative analgesia, antibiotics, and antiarrhythmic drugs under aseptic conditions used wherever possible

Best practice post-operative monitoring of animals and rapid intervention in cases of post-operative complications

Use the Rabbit Grimace Scale (Keating SC et al, 2012. PlusONE 7(9).e44437 and Body Condition Score

Continued collaboration on the MI-model

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

REDACTED

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LASA guidelines for administration of substances
http://www.verutech.com/pdf/lasa_administration.pdf

How will you ensure you continue to use the most refined methods during the lifetime of this project?



Attending NC3Rs conferences

Keeping up to date with relevant literature relating to 3Rs advances and discuss advances at our group meetings

Consider adopting any advances that will refine techniques based on the latest science and technologies

Periodically review new 3Rs information, experimental data and success and failure rate of procedures.

Ensure minimal number of animals is used for each project and discontinue animal use for projects that are not showing promising data

Design animal experiments that are robust and reproducible and ensure thorough analysis and regular review of data

Use animal data to address important scientific questions and develop appropriate mathematical models of the heart to reduce the need for animal experiments in the future

Explain the choice of species and the related life stages

It is impossible to investigate modulation of ventricular fibrillation (VF) in humans due to its lethal nature. The precise nature as to how neural and cardiac remodelling and their interaction in MI leads to increased incidence of VF cannot be studied in a controlled manner in patients as experimentation would require repeated episodes of lethal arrhythmias to explore the effects of nerve effects. Hence animal models are essential for scientific advancement relating to understanding the mechanisms involved. The use of whole animals and in vivo experiments are not ideal for studying pure effects from direct nerve stimulation on the heart due to substances in the blood and brain mediated reflexes that are difficult to control. Mathematical modelling is imperfect and still being developed to a point that would be acceptable to replace procedures requiring animals.

Hence we use the most refined model for the intended purpose of investigating the autonomic nervous system (ANS) as it allows tightly control studies without the confounding influences. Amongst small experimental animals, rabbits have the most comparable cardiac electrophysiological and ion channel characteristics to humans.





7. Basic mechanisms of chronic 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
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Mice	juvenile, adult, 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 is the aim of this project?

Overall, this project aims to investigate the mechanisms underlying neurodegenerative diseases (diseases in which the structure and function of the brain are progressively lost) in particular those involved in human and animal prion disease pathogenesis.

More specifically this will include identification of new disease strains, modelling of human prion diseases, defining interactions between the immune system and neurodegenerative pathways and could lead to identification of therapeutic targets.

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

Why is it important to undertake this work?

Neurodegenerative disease is an overarching term which covers a range of conditions in which the neurons (cells in human brain) are damaged or die and are unable to be



replaced by the body. Neurodegenerative diseases include Alzheimer's disease, Parkinson's disease, Huntington's disease and prion diseases. There is currently no cure for these diseases; they are progressively debilitating and involve both issues with movement and mental ability (dementia). In the UK, 850,000 people live with dementia disease and the cost is in the region of £26 billion/year expected to more than double by 2040. Current therapies have only a modest impact on symptoms and limited impact on disease progression. Identifying pathways and potential therapeutic targets in these diseases could aid in future treatment development.

The prion diseases, sometimes referred to as transmissible spongiform encephalopathies (TSEs) are a family of diseases in which the infectious agent lacks any genetic material (DNA) and consists of the misshapen (misfolded) and aggregated forms of one of the body's own proteins; prion protein or PrP. Some forms of the disease can be infectious and they are recognised as both animal and human diseases with potential to transmit between species including humans. They can be used as a model of chronic neurodegeneration but are also important diseases in their own right. Although the incidence of animal prion diseases such as scrapie and BSE in the UK are now low and no longer perceived as a threat, cases of variant Creutzfeldt-Jakob disease (vCJD), the human form of BSE, still occur.

Retrospective studies in anonymised appendix samples have indicated that up to 1 in 2000 individuals in the UK have evidence of abnormal prion protein, a characteristic of vCJD, this poses risks to public health because of the risk of transmission of disease through blood transfusion and surgery. We do not know if these individuals will develop disease or if they are indeed infectious at this stage. One of the studies in this project will include characterisation of prion protein in both brain and anonymised appendix material in order to assess whether this protein is infectious and will aid in future public health risk assessments. We will also continue to carry out characterisation of both human and animal prion diseases in order to identify new diseases and ascertain any risks to humans from animal disease. Characterisation of the abnormal prion protein and disease progression through the body may lead to improved diagnostic and therapeutic strategies in these diseases.

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

By the end of this project we expect that we will have identified or confirmed the strain of prion isolates and their transmission potential. In particular, we should have defined the nature of abnormal prion protein observed in the appendix of anonymised individuals. Overall we expect to have a greater understanding of prion disease pathogenesis and transmission and an insight into mechanisms of disease.

These results will be written up as open access publications, presented at conferences and shared with the general public through lay summaries and/or social media.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The immediate beneficiaries will be the scientific community with whom we share our knowledge and research materials. In the longer term, towards the end or post project, information will be shared with clinicians and public health officials if appropriate. The results of some of our studies will feed directly into public health remit and could impact on



areas such as medical decontamination and blood donation as well as confirming or informing on clinical diagnosis of human prion diseases.

How will you maximise the outputs of your work?

Publications and presentations at scientific conferences will enable the scientific community to access our results and request research materials. Data will be deposited in appropriate open access databases. Where appropriate we will work with relevant communication teams to generate press interest in our publications and engage the public in our science. Myself and my team are active in public engagement and sharing our science with the general public and we aim to produce lay summaries for both the public and for patients and families; these will be shared via our website and potentially through patient support groups.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

In a typical study animals will undergo an inoculation into the brain under anaesthesia and allowed to recover. The animals will then be monitored for clinical signs of disease until either signs of disease are observed or the study endpoint is reached at which point the animal will be humanely killed. Animals will stay in their home cages with cage mates throughout this period. From inoculation to end of study can vary anywhere between 50 to ~600 days dependent upon study.

In some cases, animals may receive an additional inoculation of material such as a bacteria or virus during the course of the study.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

No severe effects are expected.

Development of clinical signs of disease is required for a number of scientific objectives to be met; these can include weight loss or gain and changes in movement and behaviour. We have a well defined scoring system in place to recognise when these signs occur and allow clear recognition of when the humane endpoint is reached. Animals are only allowed to show clear defined signs of disease for 2 out of 3 weekly scoring sessions, when this occurs or if significant deterioration occurs, the animals are 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 species)?



The maximum severity for these studies is moderate and it is estimated that up to 80% of the animal will experience this. Primarily this is due to the method of inoculation (into the brain) and the appearance of clinical signs of disease. The other 20% will be sub-threshold/mild as they won't undergo inoculation via this route and/or are used for breeding purposes.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

The focus of this project is to study disease characteristics and progression of prion disease as well as mechanism of diseases. This cannot be done in human cases of disease. Laboratory techniques (referred to as *in vitro* techniques) methods or lower organisms such as invertebrates cannot replicate the complexity and biological context of real disease.

What was your strategy for searching for non-animal alternatives?

At present there are no non-animal alternatives which are able to measure infectivity or disease characterisation. Biochemical and pathological methods can only measure certain characteristics and are generally only available post-mortem in human and animal cases.

We utilise *in vitro* techniques where possible to assess protein misfolding and in a current study are using one such *in vitro* technique, referred to as protein misfolding cyclic amplification (PMCA) to amplify samples and identify positive isolates for inoculation into our experimental animals. In this way, we hope to decrease the number of transmission studies which would result in a negative result and reduce the number of animals required overall.

Why were they not suitable?

Currently *in vitro* techniques cannot tell us about disease progression and how it moves through the body. Although we will endeavour to use them a screening step before animal studies, they can give rise to false positives and are not suitable/compatible with all disease strains or tissues of interest.

Reduction

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



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

Animal numbers are estimated on our current usage and projecting anticipated studies.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The experiments are designed so animal studies are kept to a minimum, this includes the use of *in vitro* screening techniques where possible. Numbers are continually reviewed in order to reduce them where possible.

We consult with an in-house statistician during experimental design to minimise the numbers of animals required while still providing meaningful data outputs.

The use of animals is restricted to critical samples or situations where alternatives would give ambiguous results.

What other measures apart from good experimental design will you use to minimise numbers?

We use both males and females as a matter of course in our studies and tissues and data are shared with other researchers where appropriate or possible.

Any animals that have been used for breeding also have tissues collected. These tissues can be used as negative controls, for use *in vitro* studies (reducing our need to breed specifically for this purpose), for optimising certain laboratory procedures and for training purposes.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

In these studies wildtype and transgenic mice (mice in which their DNA has been altered) will be used because prion disease progression in mice closely resembles that observed in higher species such as sheep and humans. In some cases, the disease will remain subclinical and no overt signs of disease can be identified in the animals. Where animals do proceed to the clinical signs of disease we have a well-defined clinical coring system in place. The use of this system prevents unnecessary suffering and is reviewed as new data becomes available.

Inoculations are performed under anaesthesia and animals are expected to recover quickly after the procedure. All animals are monitored to ensure that they are not in distress.



We utilise *in vitro* approaches where possible but these can require GA animal tissue as a substrate. In these cases, animals do not undergo any procedure and are humanely killed as young adults. The use of *in vitro* screening had the potential to allow us to decrease the number of animals undergoing a transmission study.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

In order to study and characterise prion diseases we must study the disease process. Less sentient species cannot provide us with the relevant information or are not suitable hosts. Young adult animals are used as they provide us with the most relevant information and can be compared against an archive of previous studies avoiding us having to repeat studies in younger mice.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Over the past project licenses that I have held or been working under we have made continual refinements to our studies. These have included increased monitoring of animals where required, continual training of staff in clinical monitoring of animals, environmental enrichment and ensuring that we avoid single housing animals where possible. Where animals are required to carry out specific tasks, we ensure that they are familiarised with their environment beforehand.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow a variety of best practice guidance including:

Guidance on the operation of the Animals (Scientific Procedures) Act 1986

Local guidelines for specific procedures e.g. blood sampling

ARRIVE guidelines

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I read the NC3Rs newsletter and regularly check their website for news and updates. I am a member of an AWERB and also receive updates through that. I ensure my group are aware of the latest updates and that all are aware of NC3Rs and Understanding Animal research websites.

My group consults and uses ARRIVE guidelines when preparing studies/manuscripts. We also have a close working relationship with the animal unit staff and local NVS.

Explain the choice of species and the related life stages

The major aims of our studies centre on characterising prion disease and disease progression. Mice are used as prion disease in experimental mice closely reflects the disease in higher species such as sheep and humans. In particular, mice can show clinical signs of disease, dementia and have the same disease characteristics present in their brains (vacuolation and presence of the disease associated abnormal protein). These characteristics are not present in lower order species such as invertebrates or fish.



We tend to use young adult mice in our characterisation studies as these allow experimental procedures to be carried out more efficiently and we have comparable data in this age. Young and/or aged mice may be used if required to address the effect of age on a disease process.



8. Behavioural neuroscience in transgenic zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Zebra fish	juvenile, adult, neonate, embryo

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Under this licence, we want to breed and maintain genetically modified zebrafish lines to provide larvae for experimentation. These experiments will investigate how the brain of this animal produces and modifies movements.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 ability to move in a controlled and directed manner allows us to look for food, escape predators and dance the boogie-woogie. These movements are produced by precise sequences of activity in our muscles that are started by neurons in our brain and our spinal cord. Even though this process is central to how we move, it is still unclear how the brain does this. We use the larval zebrafish to study this process, as many of the brain structures involved can be found in its brain. We can much more easily study how these brain areas allow the fish to move, as we can use a large number of powerful tools to record and change the activity in specific portions of the brain.

One such brain area, the olivocerebellar system, can be found in all higher vertebrates. It is thought but not yet proven- that it receives information from the brain on what the



muscles have been instructed to do, which it then compares with information on what the muscles are actually doing. This allows it to see whether the animal is moving exactly according to plan. The importance of this has been seen in many patients who develop difficulties when this brain structure is disrupted: poor balance, tremors, and an inability to perform rapid movements. Treatment options for these patients is very limited, involving mostly rehabilitative exercises. While the causes are varied, a greater fundamental understanding of the olivocerebellar system will help future efforts to understand and remedy these conditions.

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

The main outputs of the project will be:

Peer-reviewed publications: in these we will share our findings with the academic community;

Datasets: the data underpinning the publications will be shared freely online;

Transgenic zebrafish: these are powerful tools to address a host of questions in the biomedical research field. They will be shared with any interested party (by supplying the researchers with the embryonic offspring of the relevant adult fish).

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

This project will use cutting-edge tools to investigate fundamental questions in neuroscience. Our findings will benefit the global motor systems and zebrafish research communities, and will help researchers closer to home by providing training and facilities. Motor systems research: Our experiments, which would be extremely challenging in other model organisms, will produce important results, which will be shared in publications and at conferences with data shared online at publication. We will inform researchers working on motor systems globally:

computational biologists can use our insights to update their models on these circuits, and experimental scientists can use our insights to inform their experimental design.

Zebrafish researchers: We will characterise the expression patterns of existing transgenic zebrafish, and develop new ones. These lines are powerful tools in the investigation of the function of specific cell types in neuroscience, developmental biology, and other biological fields. Our results will therefore benefit other zebrafish researchers across the biomedical field.

Project personnel: The personnel working on the project will benefit from the research as it will give them the opportunity to develop new skills and further their training to prepare them for a successful career at the forefront of science and beyond.

Further 3Rs agenda: The larval zebrafish is rapidly becoming a premier experimental model for biomedical research. Our project will help strengthen this position further in the UK. We will help achieve the UKRI's stated aim to advance further improvements in humane animal use in research through partial replacement. For other researchers aiming to do the same, this project will provide an example of a clear pathway towards that goal.

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



We will ensure maximum benefit by sharing all our findings –whether or not they are of direct relevance to the project– informally, at conferences and in publications. The new transgenic zebrafish lines will be made freely available to any interested party.

One of the ways UKRI aims to support innovation is by fostering the development of collaborative research programmes. As part of the project, we will collaborate with researchers locally and internationally. This effort will help establish international research links, and will help foster future collaborative projects between institutions.

Our research also lends itself particularly well to be presented to a lay audience, as the output can be presented in striking visualisations. The findings are also relatable and their biomedical importance apparent. We therefore intend to organise activities we think leverage these qualities best, including an annual science festival, and an undergraduate student-led project to develop activities that could be used as part of the primary educational curriculum.

Species and numbers of animals expected to be used

- Zebra fish (*Danio rerio*): 3750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 the larval zebrafish as a model organism, which due to its transparency, relatively simple body plan and availability of genetic tools makes it ideal for non-invasive experiments to uncover how the brain generates behaviour. The zebrafish brain also contains many analogous structures to its mammalian counterparts. It is therefore an excellent model system for this project, and ensures we adhere to the UKRI's rule to "Use the simplest possible (or least sentient) species of animal as appropriate to the experiment in question." We will use adults in standard breeding experiments to obtain their offspring which we will use for our experiments.

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

Typically, animals will be bred using standard protocols to obtain their offspring for experimentation or propagation of the zebrafish stock. New zebrafish might be visually screened to check for the presence of genetic markers in pre-feeding animals, or, in rare cases, genetically screened by having tissue samples taken from adults under anaesthesia for analysis. Larval zebrafish will be used in experiments, in which biological processes (typically neural activity) are observed using techniques including microscopy and electrophysiology, and manipulated using tools including pharmacology. Experiments will typically take no more than 6 hours.

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

For the most part, we do not expect adverse effects, as most animals will only carry mutations/transgenes that have not been reported to cause harm. Our breeding experiments, which may produce animals with homozygous mutations or bring these mutations in a different genetic background, may in very rare cases cause physical abnormalities. These unlikely adverse effects may cause abnormal swimming behaviours



and other phenotypes, all of which are expected to be mild. Larvae that display such defects will be humanely killed before the free-feeding stage.

General anaesthesia and fin clipping for genotyping may also produce rare adverse effects, including inflammation and infections. Squeezing the abdomen for egg and sperm isolation may in rare cases cause skin and inner organ damage.

In rare cases, the mutations/transgenes carried by the zebrafish will affect their fertility rate.

Fish will be checked daily, and all animals will be humanely killed when appropriate. In rare cases we will need to use animals carrying two copies of a mutation in a receptor that is required for skeletal muscle contraction. These animals are immobile from the point of hatching, and will therefore be humanely killed before they otherwise would have died (after 7 dpf).

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per animal type)?

Expected severities:

For protocols 1-3:

Subthreshold (>90%)

Mild (<10%)

For protocol 4:

Non-recovery (100%)

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

- Used in other projects
- Killed

Replacement

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

We are interested in how the brain produces behaviour, focusing on how movements are generated, learned, and adjusted. This research question can only be addressed by studying the animal itself. We use the larval zebrafish as a model organism; it is ideal for studying the brain, since its transparency means there is no need for surgery. It is assumed to share many functional similarities with mammals, and is therefore an excellent model system for this project. The use of this relatively simple vertebrate also ensures we adhere to the BBSRC's rule to "Use the simplest possible (or least sentient) species of animal as appropriate to the experiment in question".

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

Computational modelling: this can be useful to generate hypotheses about how the brain operates, or to test interpretations of experimental data

Cell cultures: networks of neurons grown in a dish can be used to study how these cells might interact



Why were they not suitable?

We will use the larval zebrafish to chart its neural activity and discover which areas in the brain are involved in generating movements. Computational modelling cannot reveal this, as there is currently insufficient data to generate accurate models

Cultured neurons are unlikely to recapitulate the organisation of the brain and spinal cord of the larval zebrafish. Using this technique is therefore unlikely to produce insights into how the neural circuitry in the brain and spinal cord interact to produce behaviour

Reduction

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

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

We estimated how many animals were required in our experiments using standard calculations. Using common assumptions about the variability of our data, we estimated we needed more than, or equal to, 9 animals per experiment. In some experiments, where we compare a biological process before and after a manipulation, we found that we need at least 15 animals per experiment to ensure we have confidence in our findings.

We expect to be performing 3-7 full experiments per week. If we perform an equal number of descriptive and manipulation experiments, we will require 60 pre-feeding animals per week. For the 5year duration of the project that makes ~15,000 animals. From experience, we know that ~20% of all offspring can be used in experiments. This is because each cross typically results in a large number of embryos, most of which will not be the right genotype (these will be killed before they reach 5dpf using an SK1 method), and some of which will die of natural causes. This suggests a total number of offspring required of ~75,000. In order to obtain these numbers, we require ~1,500 crosses. Assuming 15 different genotypes will be used for the project, this can be achieved with 2 holding tanks per genotype, with ~25 fish each. Assuming a mean lifespan of ~1 year per fish, this will require 3,750 fish.

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

Advice was sought from previous PPL applicants. Furthermore, the NC3Rs' Experimental Design Assistant was consulted, and dedicated software was used to do power and sample size estimations (G*Power, see reference).

Reference:

Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods*, 39, 175-191.

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

For our breeding and maintenance procedures, we will carefully adjust the number of animals we use according to our requirements and the constraints posed by the animals. For instance, the number of breeding animals for each mating will be tailored to the



number of offspring we require from them for each experiment; the fertility of each particular stock of zebrafish will also be taken into account (i.e., a stock that has a low fertility rate will need to have its numbers increased). This means we will not use more than the required animals for each experiment; nor we will use too few, which would not allow us to do statistically rigorous work. Furthermore, we have done power analyses to estimate required sample sizes for our experiments on larval zebrafish.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project. Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project aims to understand how the brain produces behaviour; the use of animals therefore cannot be avoided. We will use the larval zebrafish as a model organism, which due to its transparency makes it ideal for non-invasive imaging. Its brain is assumed to share many functional similarities with mammalian homologues. It is an excellent model system for this project, and ensures we adhere to the UKRI's rule to "Use the simplest possible (or least sentient) species of animal as appropriate to the experiment in question." For our experiments we will use well-established protocols to breed and maintain transgenic zebrafish and prepare them for experiments, ensuring minimal harm and distress. GAA passports for each line are established on a local level, frequently updated with new information, and include details relating to changes to the phenotype, breeding performance, colony management, mortality rates etc. A list of the GAAs kept at the establishment plus the GAA passports are held with the PPL, and will be provided to the Home Office Inspector on request.

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

In order to generate transgenic larval zebrafish, we need to use animals that have reached sexual maturity. The use of adult zebrafish is therefore required. Furthermore, the experiments described in protocol 4 use the earliest life stages that produce behaviour similar to adult zebrafish, namely between 5 and 7 dpf.

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

When using anaesthetics, type and depth of anaesthesia will be carefully selected and monitored in consultation with the NVS. Following fin clipping in >5dpf fish peri-operative analgesia will be provided; agents will be administered as agreed in advance with the NVS. The procedure will be carried out using sterile equipment, and the site and amount of tissue removal for genotyping will be such that there is no compromise to normal swimming. Infections can result from fin clipping (<1%) or from damage to scales or loss of mucous surface from swabbing. Any fish exhibiting any abnormal behaviour or signs associated with infection will be humanely killed.

When we create GA founders, embryos will be assessed for morphological phenotypes before the stage of independent feeding using standard light and fluorescence microscopy, and any showing morphological abnormality not required for the scientific purpose would



be humanely killed. Some genetic alterations may result in a harmful phenotype during post-hatching development evidenced as failure of larvae to inflate the swim bladder, difficulty swimming, altered morphology, failure to feed or breathing difficulties. If fish exhibit any of these adverse effects they will be killed immediately by a humane method. On occasion (<5%) late onset mutations in more mature stages may lead to lines of fish with mild lordosis (defined as less than 20% curvature of the spine), poor growth/body condition or difficulty swimming. Any fish showing harmful phenotypes will be humanely killed as soon as the phenotype is visible. During our experiments on larval zebrafish we will monitor the heart rate and blood circulation of experimental animals, and will check animals for signs of injury to ensure there are no signs of distress or harm. Any fish showing these signs will be humanely killed as soon as possible.

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

We will use well-established protocols to breed and maintain transgenic zebrafish, ensuring minimal harm and distress. These protocols have been described by the Zebrafish International Resource Center, University of Oregon, USA, sourced from "The Zebrafish Book", see reference.

Reference:

Westerfield, M. (2007) THE ZEBRAFISH BOOK, 5th Edition; A guide for the laboratory use of zebrafish (*Danio rerio*), Eugene, University of Oregon Press. Paperback.

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

The NC3Rs is the pre-eminent UK-based scientific organisation dedicated to replacing, refining and reducing the use of animals in research and testing. We will monitor the NC3Rs' output to stay aware of the latest developments in animal welfare, and implement these whenever possible.





9. Biobehavioural basis of vulnerability to impulsive/compulsive spectrum disorders - Optimisation and service protocols

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Rats	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 is the aim of this project?

To optimise procedures and produce GA rats for use on other project licences held by the group.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 use the optimisation protocols in pilot studies to optimise procedures and combinations of procedures to ensure they are as refined as possible while retaining scientific validity.

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

The expected outputs will be refinements to procedures and experimental designs and demand matched production of genetically altered rats.



What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The data obtained from the optimisation protocols will be used to inform experiments conducted by members of the research group using the other project licences held by this group. Therefore data produced from the use of these protocols will primarily be used by members of our research group. However where appropriate refinements will be published and shared with the scientific community in accordance with the principles of the 3Rs.

How will you maximise the outputs of your work?

Refinements to procedures and experimental design will be published and discussed at national and international meetings.

Species and numbers of animals expected to be used

- Rats: 1300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures. Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Rats in this project will either be bred and maintained in order to be used in the two other protocols on this licence or on our other licences.

Rats (including GA animals with no adverse phenotype) used for optimisation procedures will be subjected to intracerebral manipulations under terminal anaesthesia, with no recovery or will receive 1 intracranial procedure, and up to three surgeries overall (including the implantation of an indwelling catheter), in order to optimise manipulations or measurements of brain mechanisms while the animal is performing behavioural tasks, including drug self-administration.

Rats (no more than 60%) may be exposed to behavioural procedures aiming at measuring their motivation, impulse control or other cognitive functions, for which they sometimes need to be single housed and/or food deprived.

No more than 20% of the rats may receive administration of substances to induce dependent states. These may also be administered with substances that counteract these dependent states.

No more than 15 rats overall may experience experimentally-induced Parkinson's disease.

The investigation of specific cellular mechanisms requires the decapitation of the rats in a conscious state.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.



For the majority of our animals on this project (75%), we anticipate no more than transient discomfort and no lasting harm.

The genetically modified rats lines that we will breed have no adverse phenotype.

The two optimisation protocols are designed to enable us to minimise the impact of intracerebral procedures in subsequent experiments, by optimising and refining the said procedures first in a non- recovery protocol, and then in behaving rats, always using as few animals as possible (2 to 5).

When rats undergo surgical procedures, then tend to recover very rapidly and they are provided with post-operative care, including the use of analgesics.

When rats are trained to self-administer drugs, some (up to 25%) may develop several behavioural characteristics similar to those presented by human beings suffering from a drug addiction, including lack of interest in other sources of reinforcement and associated weight loss, decrease in self-care (their fur becomes dirtier). A very small number of animals we see them self-harm in the same way that drug addicts do when they are when given extended access to heroin. This is because high levels of heroin intake can cause changes to the way nerves in the face and mouth behave: heroin is an analgesic (i.e. it affects feeling and pain perception). When rats can no longer feel the pain some start to nibble their toe nails and toes can inflict damage to their paws.

When rats have become dependent on a drug, such as heroin, upon induction of withdrawal they display typical signs of physical withdrawal, including wet dog shakes, or piloerection, but as in humans, these signs wear off rapidly (within 24 to 48 hours).

In the case of the induction of Parkinson's Disease, rats tend to present a transient lack of motivation that is associated with the neurodegenerative process of the dopamine neurons, hence they need to be supplemented by highly palatable food or feed (including by oral gavage) until they recover (between 3 and 12 days overall).

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

Non-recovery rat protocol – 100%

Mild rat protocol – 100%

Moderate rat protocol – 100%

What will happen to the animals at the end of the study?

- Used in other projects
- Killed

Replacement

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

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



It is not yet possible to achieve our objectives without using animals, and, especially, rats. Our research, which investigates the inter-individual differences in the vulnerability to develop impulsive/compulsive spectrum disorders, which result from an alteration of many complex, intricate, brain mechanisms and associated cognitive (understanding and perception) and emotional processes which we do not yet understand, even remotely, enough to be able to contemplate modelling them using algorithms or Artificial Intelligence.

The nature of our research therefore requires we use a species whose brain, cognition and behaviour are similar enough to humans to offer insights into the psychobiological basis of human neuropsychiatric conditions. The rat is by far the best species to establish the neural and neurochemical mechanisms underlying inter-individual differences in behaviour, cognition and neuropsychiatric disorders which cannot be investigated in humans.

The rat is so far the only species in which individuals have been shown to have representation of the relationship between their actions and their consequences, to establish coping strategies, to differ in impulse control and in their propensity to take drugs, even to develop maladaptive habits and compulsivity.

Most of our animals undergo long duration behavioural experiments (which last up to 14 months) in which they perform tasks for food or drug reward, and experience procedures that, provided they are fully optimised, produce only transient discomfort and no lasting harm. It is therefore fundamental to optimise these invasive procedures, and that is precisely the goal of the present project licence.

What was your strategy for searching for non-animal alternatives?

We use alternative strategies some of which involve humans, some are computer-based models and some use Artificial Intelligence. In addition, we use cell culture (i.e. cells grown in a dish in the laboratory) and ex vivo organoids (i.e. cells that grow in a dish and form into organ like structures).

Why were they not suitable?

As our objectives, which will be achieved through the use of other complementary project licences, require complex behavioural tasks allowing for the identification of individual vulnerability to compulsive disorders, we cannot rely on in vitro (non-animal) models because these cannot reproduce the integrative function of the brain that is the focus of this research.

Since these experimental procedures are designed to optimise the intracerebral and pharmacological procedures we need to use in order to achieve our research goals in rats, the purpose of the present project licence, must be carried out in the same species.

Human studies cannot allow for a lifetime longitudinal study addressing causal mechanisms of neuropsychiatric disorders, they yield only correlative data and rely very often (if not exclusively) on retrospective analyses which prevent the identification of factors of vulnerability measured prior to the development of the disease.

Reduction

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



design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

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

1000 rats will be used for breeding and maintenance of genetically modified/selected strains and wild- type controls. This will enable us to use up to 2500 such transgenic animals in the two experimental project licences which, alongside the present one, support our programme of research.

150 rats will be used in each optimisation protocol, with groups of 2 to 5 per optimisation, that will enable us to carry out up to 20-25 optimisations over the next 5 years.

The identification of these numbers is informed by our expertise, REDACTED.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

This project licence is designed to enable us to reduce the number of animals being used in subsequent experiments under our other project licences.

Here, for each optimisation strategy, wherever possible, we implement experimental designs that minimise noise in our behavioural or biological measurements and that are always combined with state of the art statistical analyses.

What other measures apart from good experimental design will you use to minimise numbers?

We will use efficient breeding and use both male and female offspring in subsequent experiments carried out under the authority of our other project licences. We will rely on computer modelling or in vitro assays prior to optimising procedures in a non-recovery setting. We will eventually rely on very small samples (approx. 2 to 5 animals per group) to further optimise the procedures in behaving rats.

Together the incremental strategy designed for this breeding and optimisation licence will enable us substantially to optimise the number of animals we plan to use in our overall programme of 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?



In this project we will use several animal models and methods, the combination of which will enable us to identify the factors of vulnerability to develop compulsive disorders across a wide range of conditions and their biological basis in the brain.

Our methods mostly rely on awake, freely moving animals performing complex behavioural tasks for rewards (e.g. food, or drugs of abuse) and subject to invasive recordings or manipulations of the brain while performing these tasks.

We are fully committed to minimise the cumulative severity of all our procedures as much as possible and have been constantly refining all our procedures in order to minimise the distress to which our animals are subjected.

We will use models of inter-differences in impulse control (high impulsivity trait), Obsessive Compulsive Disorder, drug (alcohol, cocaine, heroin....) addiction and Parkinson's Disease.

We have, over the years, refined our models so that we can study the psychological and biological basis of profoundly debilitating and distressing disorders in humans such as OCD or addiction, with minimal adverse consequences in our rats.

In the case of OCD for instance, rats tend to display compulsive behaviours for no longer than 1 hour per day, and these have overall no other negative consequences. To study Obsessive Compulsive Disorder, we primarily rely on a procedure that requires rats be food deprived to 80% of their free feeding body weight. These rats are absolutely fine and display no behavioural or physiological signs of distress.

For drug addiction, we implemented procedures that enable us to measure the compulsive nature of drug seeking and taking that characterises addiction (in other words the drug is used despite disastrous negative consequences for the user and their relatives/carers) without overall harm to the animal. To study drug addiction, rats initially trained to self-administer drugs through an indwelling catheter implanted into their jugular vein. We have refined this procedure so that it lasts no more than 10 minutes and rats recover very rapidly with no signs of distress. All our surgeries are performed under aseptic conditions and rats are given appropriate analgesia prior to, during, and after the surgery.

For Parkinson's Disease, the procedures we use enable the rats quickly to recover from the initial motivational effects of the sudden development of the condition and ensure they do not develop too profound a motor deficit.

We use a wide array of methods in our research. One is the testing of rats in behavioural tasks in which they are given the opportunity to solve puzzles, work (press levers) to obtain food rewards. These tasks, that are designed to investigate specific psychological or behavioural mechanisms, are not regulated, meaning they do not cause any distress or harm to the animal. However, it can be the case that rats have to be slightly food restricted to engage with the task. In that case food is delivered every day following the behavioural session. Food is given in quantities large enough to maintain the animal body weight between 90 and 100% of their free-feeding weight. It is also better for the animal's health as there is strong evidence that as in humans, free-feeding in rats results in decreased longevity.

We will use systemic administration of drugs either through experimenter-delivered injections, or via previously implanted subcutaneous minipumps or slow release formulations (like some forms of pills in humans). In this case, each drug will be prepared with double distilled water or sterile vehicle, in autoclaved glassware and subsequently



filtered prior to use. Dosing procedures 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 and is the minimum consistent with the scientific objectives. We have implemented specific methods to deliver intraperitoneal injections to rats without restraining and stressing them and only highly trained researchers perform these injections.

We will use blood sampling procedures. In that case no more than 10% of total blood volume in 24hrs and 15% of total blood volume in any 28-day period will be taken.

We will use methods designed to measure or manipulate the activity of specific brain areas while rats are behaving, and/or expressing OCD, drug addiction, impulsivity. For this, we need to insert probes, electrodes inside the brain that can be connected to external devices that enable the measurement of brain function. We also rely on pharmacological manipulations of brain functions and for this cannula are lowered into the brain and we use injectors to infuse tiny amounts of drugs that interfere with a specific brain mechanism inside the brain. We will also use the technology of virus-mediated expression of genes in the brain to measure of control brain function. For all these methods, the surgical procedures are all carried out under aseptic techniques and the rats are habituated to be connected to external devices and are always free to move when connected. We use the least invasive methods drawing in fast evolving state of the art techniques and systematically ensure to procedures are optimised and refined (which is the very purpose of this project licence) before using them on larger cohorts.

When rats are food restricted, they often need to be single housed as the dominant would otherwise experience no food restriction and the expense of the subordinate. Similarly, when rats have received intrajugular and/or intracranial implants, they often need to be single housed in order to prevent them from playing with, and damaging each other's implant.

Isolated animals tend to gain more weight than controls; they are more responsive to stimuli predicting reward (in specific behavioural tests) and are hyperactive as measured by specific behavioural tasks. Additionally, we have shown that rats raised in an enriched environment are more vulnerable to develop compulsive disorders.

Overall, we are geared towards optimal refinement, from our choice of animals, to our methods, procedures and skills. First, and foremost, the present licence will enable us to optimise and refine on small groups of rats the intracerebral and pharmacological procedures that will subsequently be used on large experimental groups. The present project licence is therefore testament to our commitment to refinement.

Additionally, I make sure that we maintain our high standards in order to ensure all our refinements are actually implemented. Thus, I review all procedures and skills of the licenced researchers working in my laboratory, under my supervision, regularly and discuss project licence-related matters at each of my weekly lab meetings.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Rats are the least sentient organism that enable the measure of inter-individual differences in the behaviours of interest with some individual displaying behavioural manifestations that have high heuristic value with regards to the Impulsive/Compulsive Spectrum disorders we are investigating.



What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

When rats have to be singly housed for the purpose of protracted self-administration or behavioural training, not only are they handled at least twice a day, during behavioural training, at least 5 days a week (in average), but they are also brought to test boxes several hours per day, at least five days a week.

We have experimental evidence that these housing conditions, as opposed to so-called enriched environments, do actually protect rats from developing disabling compulsive disorders such as addiction to cocaine. Thus, our recent work on environmental enrichment has actually shed new light on the rather anthropomorphic view of the benefit of enriched environments for rodents, as it is likely more difficult to define what is an enriched environment for rats than it is for humans, for whom the definition is very much specific to each individual. Indeed, we have, in a very challenging environment, demonstrated that rats raised in a highly enriched environment are less likely to engage in high levels of cocaine self-administration than rats raised in standard conditions, but instead demonstrated higher vulnerability to switch from controlled cocaine intake to addiction. Thus, of all the 48 rats (24 from a standard condition, 24 from an enriched environment) trained to self-administer cocaine for over 80 days in this experiment, 6 became addicted to cocaine, and they were all from the enriched environment.

For repeated intraperitoneal injections we ensure that the smallest needles and volumes of pH-neutral injections are used and use procedures we have developed and refined, and validated by our Named Veterinary Surgeon, whereby stress is minimised delivering these injections without restraining the animal.

Each of the rats on the optimisation protocols on this licence will undergo neural manipulations (brain surgery) and/or implantation of intravenous catheters for long-term self-administration of drugs of abuse. We take great care to minimise suffering following surgical procedures and minimise the risk of infection and/or catheter damage by using the most elaborate techniques (the present licence is actually designed further to optimise each bespoke intracranial manipulation to further decrease its potential impact on the animals) that have been developed and constantly refined in collaboration with the Named Veterinary Surgeon.

We routinely administer peri-operative analgesia (i.e. pain killers after surgery) and use scoring sheets to monitor animal welfare for a minimum of three days post-surgery. The specific analgesics used varies between different types of experiment and the strategies we have now in place to ensure continuous analgesia through the perioperative period have been designed with the Named Veterinary Surgeon.

We have been investing a lot of time and efforts in refining our skills, techniques, procedures and equipment, and we will continue to do so over the next five years. Over the past years, we have developed and refined, together with our Named Veterinary Surgeon, a scale to enable the daily monitoring (using an electronic reporting system we have also implanted and refined) of the status of the back mounted cannulae ports in rats trained to self-administer drugs. We have also substantially improved our aseptic techniques adopted from the principles of the Laboratory Animal Science Association (LASA) guidelines, to comply with our high throughput requirements.



We have progressively engineered a better catheter for self-administration experiments. Relying on a stepwise, iterative empirical strategy, we have decreased the size of the mesh that is inserted under the skin to secure the port, in order to decrease the surface it occupies under the skin, hence the risk of irritation and physical damage to the surrounding tissue. Having verified, in collaboration with the Named Veterinary Surgeon that this change yielded positive outcomes in terms of damage to the skin around the port, we then increased the rigidity of that mesh to stop it being grabbed and stretched by the rats, which on some occasions resulted in local irritation. Having observed another improvement, we then decreased the diameter of the port cannula (tube) which reduced the overall size of the device.

We predominantly use Sprague Dawley rats, and not the Lister-Hooded strain which were historically used, as the former demonstrate fewer skin lesions, and tolerate the effects of long-term drug exposure better than the latter. The very low incidence (actually the absence) of adverse effects around ports in 200 rats that had the new monitoring procedure illustrates the improvements made by my laboratory and we will endeavour to further the refinement of this, as well as all the other procedures used in my laboratory. All improvements will be disseminated as widely as possible.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

REDACTED

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I have several lines of information that enable me to stay informed about advances in the 3Rs in order to implement them effectively.

First, I have registered to the NC3Rs newsletter and follow them on Twitter.

Secondly, as all the project licence holders at our establishment, I receive tremendous support from the staff at the establishment, and I receive regular critical updates from the Named Information and Compliance Officer to which I pay the utmost attention and that I share with all the members of my lab.

I hold project licence-related workshops at least twice a year with all the members of my laboratory to discuss the changes in procedures.

I have an excellent working relationship with the animal care staff in my animal facility, which facilitates the implementation of advances in the 3Rs.

Explain the choice of species and the related life stages

The majority of individuals in the world are exposed to stress, or use drugs recreationally, may it be alcohol, tobacco..., sometimes itself as a means to deal with stress, a so-called coping strategy, we are not equally capable of developing adaptive coping strategies or to maintain a recreational, controlled use of drugs. The individual vulnerability to lose control over coping strategies or drug use, which results in Compulsive Disorders such as Obsessive Compulsive Disorder or drug addiction, remains poorly understood. This prevents the development of novel, more effective therapeutic or preventive strategies for these debilitating disorders that affects millions of individuals worldwide. Our research,



which relies on two complementary experimental project licences alongside this one, interests itself with the environmental, psychological, neural and cellular factors that contribute to this individual vulnerability to develop Impulsive/compulsive Spectrum Disorders.

This research is only possible with the use of animals because human studies (e.g. brain imaging studies) are useful but can only provide correlative data that do not address causation and short fall of identifying the detailed mechanisms in the brain that support the vulnerability to develop Impulsive/Compulsive Spectrum Disorders. Furthermore, it is not ethically possible to study the genetic and/or environmental factors that underlie predisposition to, and the development of, neuropsychiatric disorders in humans.

Similarly, it would not be possible to develop new treatments for brain disorders without testing them in animal models first. In vitro models (e.g. rat brain slice preparations) or computer simulations cannot be used because the modelling of behaviour in these systems is not sufficiently advanced.

Thus, we use rats because they are the least sentient species which allows researchers to investigate marked inter-individual differences in sophisticated behaviours that capture core features of these neuropsychiatric disorders. Additionally, the brain circuitry implicated in many neuropsychiatric disorders is highly conserved between rats and humans.

In order to uncover the biobehavioural basis of the vulnerability vs resilience to ICSDs across the lifespan, we will carry out investigations, deploying large array of intracerebral measurements and manipulations on juvenile and adult wild-type and genetically modified rats. It is therefore important that such procedures, which are invasive, are optimised prior to being deployed on large experimental cohorts of rats. The purpose of the present project is therefore the breeding and maintenance of genetically modified rats as well as the optimisation of intracerebral techniques and pharmacological manipulations.

The identified need for optimising our procedures stems from our commitment to animal welfare. Indeed, most of our animals run in long-lasting behavioural experiments in which they perform tasks for food or drug reward, and experience procedures, when fully optimised, that produce only transient discomfort and no lasting harm, achieved by a constant refinement of administration techniques by well- trained personnel.

Any adverse effects are discussed with the Named Veterinary Surgeon. If these cannot be quickly ameliorated then animals are killed to prevent suffering.





10. Biomechanics and development of cranial sutures in lagomorphs

Project duration

1 years 9 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Rabbits	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 is the aim of this project?

This project aims to advance knowledge of the biomechanical role of cranial sutures (soft connective tissue joints between the skull bones) in overall skull function and development, by investigating how the patterns of cranial strain are influenced by the patency of sutures and suture-like joints.

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

Why is it important to undertake this work?

Craniofacial sutures are unique joints found in the skull and are important sites for bone growth during development, and for absorption of mechanical stress. The primary benefit of this research is the generation of new knowledge on how cranial sutures transmit biomechanical forces (mechanobiology) within the vertebrate skull, and is fundamentally important to improve our understanding of how craniofacial sutures and skeletal muscles influence each other in developing skulls.



The major goal of this study is to measure and compare forces exerted on a skull to the strain imposed by these forces on cranial sutures across the development of young rabbits with normal skull morphology, and those with sutures which fuse prematurely. In the short-term, this will improve our understanding of the evolution and function of cranial sutures. This will be broadly useful to researchers studying evolution, biomechanics, and bone and muscle physiology and function.

The proposed research is also expected have long-term benefits for healthcare. Expected outcomes of this project include expanding our knowledge of suture development, and refining and improving techniques for biomechanical modelling of cranial sutures. This will provide benefits to the academic community through technological advances, and to clinicians who use orthopaedic devices to modulate sutural growth in patients with conditions such as craniosynostosis that affect suture development and patency.

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

The primary outputs of this research is the generation of new knowledge on the mechanobiology and development of cranial sutures, and suture-like joints. This will be disseminated through peer-reviewed publications (e.g. in broad multi-disciplinary journals like Royal Society Proceeding B) and at national and international conferences.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short-term, peer-reviewed publications and conference presentations will be used to disseminate the outcomes of this research. This will improve our understanding of the evolution and function of cranial sutures, and will be broadly useful to researchers studying evolution, biomechanics, and bone and muscle physiology and function.

The proposed research is also expected have long-term benefits for healthcare. Expected outcomes include expanding our knowledge of suture development, and refining and improving techniques for biomechanical modelling of cranial sutures. This will provide benefits to the academic community through technological advances, and to clinicians who use orthopaedic devices to modulate sutural growth in patients with conditions such as craniosynostosis that affect suture development and patency.

How will you maximise the outputs of your work?

This research involves collaborations with academics and clinicians (plastic surgeons) from both the UK and US. The research will be disseminated both locally and globally through publications and conferences, as well as reaching a broader network through social media, such as Twitter and blogs. These methods will enhance the reach of the publications as demonstrated with Altmetric and traditional metrics.

Species and numbers of animals expected to be used

- Rabbits: 40

Predicted harms



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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Rabbits will undergo minor surgery to implant rosette strain gauges across cranial sutures, and small radio-opaque beads implanted in the skull bones to measure the movement of bones (kinematics) during x-ray biplanar videography. After recovery from surgery, strain, muscle activity and kinematics, will be recorded during normal behaviours such as feeding during the course of one day. At the conclusion of this study, rabbits will be humanly killed with a schedule 1 method. These rabbits will then be used to collect muscles properties data through dissections, and CT scans to build finite element models.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The most likely source of adverse effects is stress after surgery, or during transportation to, and filming in, the biplanar x-ray facility. Complications and infection after surgical implantation of the gauges is possible, but not likely, due to the sterile setting of the surgical room, and the after-care drugs including analgesia and antibiotics (e.g., Baytril, ketofen, famataide and normal saline, or as advised by veterinary personnel). Rabbits will be habituated to the box that they go in for the x-ray bi-planar imaging to reduce the likelihood of stress during transportation and data collection.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

Moderate severity will be expected for all rabbits because minor surgery is involved.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Replacement of animal models is not possible, as a major goal of this study is to determine bone strain and motion during natural behaviours. *Ex vivo* and *in silico* simulations of cadaver specimens can only indicate the range of motions that are physically possible based on joint shape, not the motions actually employed by a living animal, and can only estimate the physical strains experienced during simulated motions. Because humans suffer from craniosynostosis, it is essential to study organisms that have a similar etiology and phenotype. Studies of these rabbits with craniosynostosis could lead



to preventing craniofacial growth disturbances and decrease postoperative morbidity in infants with premature suture fusion.

What was your strategy for searching for non-animal alternatives?

Non-animal models are not available.

Why were they not suitable?

The technology of virtual biomechanical models are not sophisticated enough to replace *in vivo* data, and cannot measure real motion or strain experienced by an animal during natural behaviours. These data require direct measurements taken from live animals. These data will be integrated with imaging and *in silico* biomechanical modelling for validation, as described in the project plan.

Reduction

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

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

No preliminary or prior data are available to determine the number of animals required to complete the study. Judging sample size is difficult because this study does not test a statistical hypothesis, and only needs sufficient sample sizes to assess intraspecific variation in morphology and function.

It is unlikely that three (3) individuals will be sufficient to distinguish between intraspecific and interspecific variation in morphology between a group that varies so much due to the condition of craniosynostosis. Therefore, we have estimated that 10 animals from each group (Juveniles with craniosynostosis; juveniles without craniosynostosis; adults with craniosynostosis; adults without craniosynostosis) will be required to collect high quality data. This number also accounts for up to 20% potential losses of rabbits across the duration of this study. We will carry out a pilot study after three (3) rabbits are tested to see if the minimum number of animals can be reduced, and again conduct an interim analysis after six (6) rabbits.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

No preliminary or prior data are available to determine the number of animals required to complete the study. Data collection for multiple outputs (strain, muscle activation, bite force and kinematics) can be collected for each animal during the same experiment. This will reduce the number of animals we need, and make the subject specific biomechanical models more accurate with input data from the same individual. We will carry out an interim analysis after six (6) rabbits are tested to see if the minimum number of animals can be reduced.

What other measures apart from good experimental design will you use to minimise numbers?



We will conduct a pilot study to measure strain, bite force and muscle activation in 3 adult rabbits.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Strain gauges are the only method to collect *in vivo* strain during function, and can also be used to validate the finite element models that will be used for future hypothesis testing without the use of animals. However, to know if these models accurately represent reality, the *in vivo* data is essential for validation. The x-ray based methods are currently the most precise and accurate techniques for measuring 3D, *in vivo* bone motion. The required surgery for these methods will be done using general anaesthesia and analgesia to minimise any pain or suffering.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Rabbits experience naturally occurring familial craniosynostosis, where the cranial sutures fuse too early during development. Suture defects must be artificially created to investigate this question in any other species of mammal. Therefore, these rabbits are a better model for modelling the biomechanical effects of suture fusion than an animal with an artificially fused suture, and are the most similar non-human model to naturally occurring craniosynostosis in humans and require less surgical manipulation.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals will be monitored after surgery for post-operative care and pain management. If irritation is caused by the strain gauge wires, shorter, more insulated, and/or more flexible wires will be considered. Veterinary advice will be sought for any rabbit giving cause for concern and additional analgesia may be given if appropriate.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

No experimental data using these procedures on these species have been published. Similar data only exists in studies on rats. Therefore, these publications have been used for best practice guidance.

How will you ensure you continue to use the most refined methods during the lifetime of this project?



I will continue to be involved in training programs and attend seminars. I will also enrol any PhD students and postdoctoral staff in the Personal Licence training who join the project, and supervise their training in the protocols.

Explain the choice of species and the related life stages

Craniosynostotic rabbits are the only existing colony (of any species) affected by familial craniosynostosis in the world. Suture defects must be artificially created to investigate this question in any other species of mammal. Therefore, rabbits are the most similar non-human model to naturally occurring craniosynostosis in other mammals and require less surgical manipulation. Because humans suffer from craniosynostosis, it is essential to study organisms (like these rabbits) that have a similar etiology and phenotype. Non-animal models are not available. Similar data only exists in studies on rats. Rabbits are much larger than rats and the biomechanics of their skull is likely more similar to a humans due to non-allometric characteristics of larger skulls and muscles. To measure the effects of suture fusion, we will collect strain over sutures during growth, as juveniles, and as adults when the skull has matured and the sutures have fused.





11. Blocking senescence in ageing

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

To assess the importance of senescence in ageing phenotypes and to test the biological effects of preventing senescent cell accumulation on ageing amelioration.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 to understand how and why ageing develops and what is the importance of the accumulation of old (senescent) cells in age-related pathologies (such as Alzheimer's, sarcopenia, heart failure or cancer). Also, we will study new therapeutic



approaches to ameliorate ageing, based on the results obtained REDACTED, which identified several potential anti-senescent strategies.

We will use existing mouse models that recapitulate normal human ageing as well as in premature ageing syndromes, such as progeria. We may also use sublethal whole body irradiation as a known protocol to induce senescence in cells and thus accelerate tissue ageing. This will help understand the mechanisms responsible for normal and pathological ageing and their associated diseases, and could explain why some people age better than others and have longer lifespan. Our experiments will facilitate the design of new tools to study ageing and, eventually, to prolong lifespan/healthspan in humans.

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

We expect to determine whether nanoparticles can be used in vivo to eliminate senescent cells and thus ameliorate age-related pathologies. Also, we want to determine whether these nanoparticles could be used as diagnostic/prognostic tools in ageing. On the other hand, we expect to obtain information on other chemical compounds that can stop senescent cell accumulation in tissues and have an effect of mammalian lifespan/healthspan. This will lead to new publications and, potentially, provide the basis for clinical trials.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Studies of ageing in mouse models are indispensable and complementary to other research experiments. These models are important to understand how and why ageing develops in an organism and which is the impact of old cells in the development of age-related pathologies such as cancer. We would like to use existing mouse models that will recapitulate what is observed during normal human ageing, as well as in premature ageing syndromes such as progeria. This will help us to understand the mechanisms responsible for normal and pathological ageing and their associated diseases and may help us to explain why some people develop age better than others and have longer lifespan.

The short-term benefits of our research will impact the community of scientists studying senescence ageing and age-related diseases, including the generation of new tools for senescence research. Also, the researchers working on this project will benefit by being trained in state of the art techniques and protocols by leading experts. A mid-term benefit will be that companies in the commercial private sector could engage in testing the approaches suggested by our research. This could foster the economic competitiveness of national enterprises and attract R&D investments from global business. In the long term, our experiments will facilitate the design of new therapeutic tools to improve ageing and/or prolong lifespan in humans. Thus, they would benefit geriatric patients suffering age-related diseases such as cataracts, diabetes, Alzheimer's, cancer, etc., as well as those having premature ageing syndromes. Many of the diseases have no cure and could greatly benefit from anti-senescent strategies. The therapeutic tools derived from the knowledge generated in this project could have an impact on globally enhancing quality of life in the future.

How will you maximise the outputs of your work?

We have several collaborations with scientists working on the field of senescence and ageing, as well as biotechnology companies. We will use their expertise to take our results



forward and test any compounds in other models, in order to accelerate any translational applications. We will also focus on the public dissemination of our results, with publication of articles in specialist journals, presenting results in conferences, networking with focused groups and engaging a wider audience through activities aimed at the public dissemination of science (such as open days and lecture series).

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will be given drugs (by oral gavage, tail vein injection or other methods) and changes in lifespan/healthspan will be recorded by measuring a frailty index over their whole lifespan. Functional tests will be performed on some of them to assess amelioration of specific age-related symptoms (e.g. Barnes maze to test spatial memory, Kondziella test to measure muscle strength, functional imaging for heart function, etc.) and blood samples may also be obtained.

Animals will be dosed starting after 2 months of life and kept in the protocol until the endpoints are achieved. A single animal will not be subjected to all the optional steps described above. Involvement of each animal in one or more optional steps will be carefully determined to minimize stress. Animals are likely to be involved in 2-3 of these steps maximum for most of the experiments.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

In most of the steps, no adverse effects are expected, except those associated with ageing. Many compounds and interventions to be used have already been tested in mice and shown to produce minimal side effects. Some agents may cause discomfort (e.g. local injection site reaction) when given repeatedly. The likelihood of such an event is extremely low. We are not anticipating the increased appearance of tumours in the mice used in these experiments due to their shortened average lifespans. Mice may develop reduced mobility as they age, accompanied by a general reduction in organ function (which can lead to dental disease, corneal opacity, dermatitis, lameness, seizures and other age-related symptoms).

Although unlikely with the types of drugs used for the experiments described here, adverse effects (including death) should always be expected when using therapeutic approaches that have not been reported before. Thus, these new compounds will be tested in a small scale pilot study to determine toxicity and potential side effects before being used in the adequate steps.



Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

Mild/moderate for all animals (likely to be 70% mild, 30% moderate).

What will happen to the animals at the end of the study?

- Used in other projects
- Killed

Replacement

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

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

Cell cultures cannot recapitulate the response of a full organism when it comes to ageing. We did preliminary tests on flies but, although genetically similar to humans in many aspects, they are evolutionarily too distant for experiments done on them to be conclusive in terms of human applications. Non-mammalian models, such as fish or *C. elegans*, have limited interest for advanced studies due to the huge differences in the ageing processes between these species and human. Thus, this is why experiments in a mammalian model such as mice are necessary in order to bring these compounds to potential clinical trials.

What was your strategy for searching for non-animal alternatives?

All preliminary experiments to test the biological effects of the compounds to be tested in mice were done in mammalian cell cultures.

Why were they not suitable?

The relevance of cell culture models to assess organismal ageing is very limited.

Reduction

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

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

We based this estimate on the mice used during the five years of the previous licence and the power calculations performed specifically for this project. In our previous work, we tested different drug concentrations of an anti-senescent drug and we will now use the dose that showed less side effects, thus reducing the number of animals needed for preliminary tests. We have used online tools (see below) to estimate the number of animals needed to achieve significance using ANOVA tests.



What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We used the advice of in-house experts and the following online tools:

<https://www.statisticshowto.datasciencecentral.com/>

<http://www.quantitativeskills.com/sisa/calculations/samsize.htm>

NC3Rs' Experimental Design Assistant

What other measures apart from good experimental design will you use to minimise numbers?

Breeding will be carefully controlled to avoid surplus animals. Data from previous studies will be used to determine the best drug concentrations and dosage schedules. When using new anti-ageing drugs, pilot studies will be performed first to determine the best doses.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use normal and fast ageing mice. Fast ageing mice allow us to reduce the time the animals are kept in the study, but they do not represent physiological ageing. We will assess the lifespan/healthspan changes in the animals using a frailty score, such as the one described elsewhere in this document. The animals will suffer no harm or distress, other than that associated with normal ageing (preliminary experiments show that the side effects of the compounds used are minimal) or potential unknown side effects when using new anti-ageing drugs (risk of suffering will be minimized by testing these drugs first in a small pilot study).

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Although useful for preliminary studies (for instance, we first tested some of our drugs in flies), non-mammalian models are not adequate for pre-clinical studies of interventions in humans since they do not fully recapitulate human ageing phenotypes.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The animals will be checked constantly for distress as part of the frailty assessment (a test where we measure age-related parameters such as locomotion, hydration, muscle tone, etc.). Given the fact that distress signs are an essential part of the ageing phenotype, we will already be monitoring very carefully their appearance during the course of the study, due to the intrinsic experimental value of the data. Interventions will be immediate if any threshold is reached, thus minimizing the harm to the animals.



What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will use the PREPARE and ARRIVE guidelines for planning and reporting experiments, as well as the LASA dosing and blood sampling guidance.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will be assisted by the staff of the animal facility, who will inform us of any changes. We will liaise with the REDACTED's NC3Rs Programme Manager during the project. Moreover, the NIO circulates a Newsletter that incorporates up to date information from the NC3Rs website. Throughout the duration of the project, we will always look at advancing the 3Rs that we learn from the information received.

Explain the choice of species and the related life stages

Mice are the most adequate and efficient model for the study of mammalian ageing. Mice will be studied over their whole lifespan.



12. Bone loss: why it occurs and ways to reverse it

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Bone loss frequently accompanies increasing age and disease states such as arthritis, diabetes and certain cancers. This project aims to prevent bone loss in these conditions and to identify new ways to replace bone once it has been lost.

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

Why is it important to undertake this work?

Musculoskeletal diseases, involving muscles, bones, joints, tendons and ligaments, represent one of the greatest long term health care burdens worldwide. Osteoporosis causes more than 8.9 million fractures annually and 1 in 3 women over the age of 50 will experience osteoporotic fractures, as will 1 in 5 men aged over 50. Bone loss also occurs in arthritis, diabetes and in bone marrow cancers such as multiple myeloma. In these



disorders, bone loss occurs faster than it can be accrued leading to complications of a weakened immune system, bone pain, deformity and susceptibility to fracture.

This project centers on ways to prevent bone loss and/or replace bone once it has been lost. There is a significant unmet clinical need for bone forming agents. We aim to understand how bone loss and growth is triggered with a view to applying this knowledge to develop new therapeutic strategies to treat some of the biggest issues for musculoskeletal health such as osteoporotic bone loss.

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

Autoimmune disorders affect up to 1% of the population and present an ongoing burden on clinical resources with joint destruction presenting the need for costly revision surgery in advanced cases. Anyklosing spondylitis has the added complication of developing inappropriate new bone formation as well as joint destruction and gut inflammation. Of even greater prevalence is osteoporosis and whilst therapies such as bisphosphonates have revolutionised its treatment, there remains the need to find ways to replace bone once it has been lost. Cancer-induced bone diseases such as multiple myeloma and metastasis to bone have a very poor clinical prognosis and ways to increase lifespan and therapeutic interventions are vital.

This project has the potential to address a number of unmet clinical needs that despite recent advances continue to elude therapeutic intervention. Additionally the discovery of novel bone forming agents and how the body normally produces them, has the ability to provide the next generation of treatment for osteoporosis – one of the largest bone disorders affecting our ever ageing population.

The earlier we can treat patients with autoimmune and cancer associated bone diseases - the better the long term prognosis will be for the individual in terms of disability and mortality.

Work into a novel osteoclast inhibitor is underway and involves the repurposing of an existing drug. This speeds the time to clinic as it has existing safety profiles making the translation pre-clinical models to human trials much faster. It is envisaged that this will take 2-3 years.

Work into ways to alter the bone marrow microenvironment and to alter stem cell fate in favour of osteoblasts is similarly underway and we have candidate molecules that control the process. We need to determine what other factors interact and to design therapeutic agents to alter the process. This part of the project is likely to require the next 3-5 years.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short to medium term, this work will benefit scientists and clinicians in the musculoskeletal field by providing new insights into the mechanisms of action in inflammatory bone diseases and cancer- induced bone changes. The multiple myeloma model we are using has previously been used for new drug development as it faithfully recapitulates the clinical features of this disease. This is an important benefit as we move towards the prevention and/or early intervention in disease in the human population from the earliest signs of symptoms – this can only be achieved if we know the initiating events. All work will be published in peer reviewed articles, online commentary and via social



media to ensure the broadest dissemination of results. There are implications for pharmaceutical and biotech companies for the re-purposing of drugs as new therapeutic options as well as the potential development of new in-house therapeutics.

The longer term implications of this work are an impact on clinical practice and potential therapeutic options. In western countries, autoimmune disorders have prevalence rates of 1% for RA and 0.25% for ankylosing spondylitis whilst the life-time risk of developing cancer is 1 in 3. These diseases are all long-term and cause early mortality. Many patients are unable to work, so the socioeconomic impact is high. One of the first line therapies to reduce inflammation is the administration of glucocorticoids yet depending on time and dose these may also be detrimental to bone.

Fragility fractures resulting from low bone mass in osteoporosis increase morbidity and mortality in adult populations worldwide. According to the National Osteoporosis Society more than 3 million people in the UK are estimated to have osteoporosis with 50 % of women and 20 % of men being affected over the age of 50. With estimated costs of £2.3 billion per annum in the UK alone osteoporosis represents an enormous burden to the health care system. With an ageing population in Europe and North America the incidence of osteoporosis and fragility fractures will increase rapidly in the future. By modulating the interplay of immune cells with bone forming cells, we aim to counteract bone destruction in osteoporosis.

Humans present with different bone related cancers that are exceptionally difficult to treat due to the location of the disease and the importance of protecting haematopoiesis. Our animal models that mimic bone cancer and cancer metastases to bone will reveal the underlying disease mechanisms and ultimately improve cancer therapy options.

How will you maximise the outputs of your work?

Via collaboration we will share our expertise in these in vivo models and their analysis of bone phenotype with the wider scientific community. Any new protocols or refinements will be published in the relevant Methods journal or within our scientific publications.

Species and numbers of animals expected to be used

- Mice: 6000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The majority of mice will undergo injections with a small percentage of mice having a surgical procedure. The aim of this project is to investigate changes in bone and as bone is slow to change, this means that the duration of experiments ranges from days up to 20 weeks after the initiation of disease.



Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Adverse effects such as pain, weight loss, inflammation and bone marrow tumours will occur in this project. Transient pain will occur post surgery (for induction of osteoporosis) for 24-48 hours and will be treated with analgesia. Swelling and redness of joints will occur in the arthritis models and will be monitored to ensure that this does not result in altered behaviour (i.e. change in walking or climbing pattern, ability to feed). Any weight loss will be monitored twice weekly in the inflammatory arthritis model and in the bone cancers; the durations of these models are 1-6 weeks. The development of bone marrow tumours is a 3 week model that causes little observable impact on the daily activities of the mice however during the final 2-3 days the mice need to be observed daily for signs of problems with 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 species)?

The severity of all protocols is set at moderate however actual severity will be mild or sub-threshold in 50% of the mice.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 application has been compiled with due consideration of the 3R's: Reduction, Replacement and Refinement. In the inflammation models, the steps leading to the development of the state of chronic inflammation are poorly understood and it is likely that multiple physiological processes are involved, including proliferation of precursor cells, activation of lymphocytes and recruitment of inflammatory cells to the site of inflammation. Inflammation-induced bone disorders are not static diseases confined to a single tissue or time point and hence modelling the effects of new therapies inevitably involves the use of whole organisms and, in particular, the use of mouse models of inflammation. However, where possible, cells from patients will be used to address questions relating to the mechanisms of action of therapeutic targets.

For osteoporosis the high incidence of bone loss leading to fractures can be better simulated in vertebrate animals rather than tissue culture as bone represents a highly complex tissue in close contact with other tissues such as blood and mesenchyme. These associated tissues have been shown to strongly influence processes of bone homeostasis and repair thus the need to employ mouse models of osteoporosis. However, as we have shown previously, co-cultures of bone forming cells with immune cells are informative in terms of bone formation signals and we use such assays whenever possible.



For the cancer models, there is no valid in vitro system for replacing the complex interactions between the immune system, cancer cells and bone in transplantation biology. Animal models for cancer development in bone constitute a major prerequisite for rapid bench-to-bedside translation of investigational anticancer therapies. Bone destruction following cancer cell invasion is induced by interaction of the cancer cells with bone and immune cells and is correlated with many complex processes such as angiogenesis, fibroblast migration and osteoclast activation which concertedly cannot be reproduced in vitro.

Likewise diabetes is a multi-organ, multi-cellular disease that is not able to be modelled in vitro especially given that certain aspects such as modifications in bone mineral density develop only in the later stages of the disease.

What was your strategy for searching for non-animal alternatives?

For the majority of our work we use in vitro tissue culture techniques to provide information about molecular and cellular mechanism(s) involved in the pathogenesis of disease or therapeutic effects. These cell culture assays will be used to assess potential therapeutic molecules in functional assays such as expression, activation, proliferation and cytotoxicity. Molecules shown to be inactive in these assays will not be examined further in vivo. This utilisation of in vitro assays is an important replacement of in vivo experiments.

Why were they not suitable?

There are limitations to the cellular complexity that can be achieved in in vitro cultures and hence the need to use in vivo models to investigate complex multi-organ disease states that vary in their presentation and cellular composition over time.

Reduction

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

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

Mouse numbers have been determined using power calculations to ascertain the minimum number of mice needed to obtain statistically significant results thus reducing the number of repeats and failed experiments.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

During design the number of animals has been determined by a combination of power calculations and knowledge of the disease penetrance in a given model. When establishing a new model or treatment pilot experiments are run to ensure that the disease is suitably mimicked and that disease severity limits are adhered to.

What other measures apart from good experimental design will you use to minimise numbers?



For the ankylosing spondylitis model, the penetrance is 80-90% in female mice and 50-60% in males – hence female mice will be used preferentially to reduce animal numbers.

Whenever possible the same mouse will be used as experimental and also as control individual. For example the LPS mediated calvarial local bone resorption model allows the use of the right calvarial parietal bone as control when subcutaneously injected with PBS, while the left parietal bone of the same calvaria when injected with low doses of LPS (<10mg/kg) can be used as experimental bone. Whenever possible, untreated control groups will be shared between treatment groups. In addition, untreated control groups will be used as a source of tissues for analysis of expression of pro-inflammatory mediators.

In the cancer model, animal numbers will be minimized by performing multiple assessments on the same animal and transplanting mice with in vivo visible cancer cells (i.e. luciferase) to evaluate the same animal at different time points rather than using several mice for different end points. Whenever possible the same mouse will be used as experimental and also as control individual. For example unilateral intra-tibial transplantation of multiple myeloma cells allows following up the cancer-induced bone disease in the transplanted leg while the other leg may be sham transplanted with PBS and used as control. Further reduction in animal numbers can be achieved with dietary manipulation to ensure that all of the test animals develop disease.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Before introducing new drugs, chemicals or substances in the treatment regimen or experimental protocol we will conduct dose-response assessment and toxicology testing pilot experiments. Pilot experiments on a reduced number of mice are regularly employed by our group and, in our experience, proved to be crucial when choosing the source (accredited supplier/company) of new diet interventions (e.g. when testing high and low fat diets from different accredited suppliers we observed that one of low fat diet produced the unwanted side effect of weight loss).

For some protocols (e.g. ankylosing spondylitis (AS), bone cancers) where some pain may be experienced by the experimental animals, we plan to run a pilot experiment in order to establish the nature, dose and frequency of analgesic administration. In the first instance, Gabapentin will be administered in the drinking water to diseased and symptomatic mice at different concentrations (3, 10, 30 mg/kg). To date, Gabapentin is considered to be one of the best analgesics for neuropathic pain.

We have introduced a number of protocols aimed at testing specific disease features in an isolated site (airpouch, calvarial bone changes, phenotyping and ageing, dose and toxicity in naive mice; protocols 2, 3, 5 and 6) thus reducing the use of multiple interventions in diseased mice (AS, bone cancer, osteoporosis, diabetes) hence reducing the suffering of



and lasting harm to the mice. By only using the disease models at an optimised dose/time, we will reduce the numbers of animals used and the degree of distress suffered.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Bone diseases primarily effect adult stages of life and the way that bone grows during embryogenesis and throughout adolescence is very different to that of the adult therefore this is the lifestage that needs to be investigated. To model human bone disease it is vital to have a vertebrate organism. Some bone work has been done in zebrafish however their immune system is not complementary and they do not develop inflammatory arthritis or osteoporosis. Additionally zebrafish exhibit properties of bone that humans do not share i.e. bone regrowth following amputation.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Experiments will, where possible be refined through the use of imaging technologies so that maximum information can be obtained from each animal used in a study. In all protocols we will aim to utilise approaches that minimise the animal's potential suffering, i.e. effective analgesia, improved surgical technique and alternating drug dosing regime to reduce volume and frequency of administration. Analgesia will be provided following surgery in all cases.

In case of intra-tibial transplantation we will make every effort to minimise the length of the procedure to minimise anaesthesia time as well as giving analgesics to minimise pain. Analgesia pilots for disease pain are included in Protocols 4 and 8 (AS and bone cancer). If there is no response within 24 hours to the administration of analgesia, the animal will be sacrificed via a Schedule 1 method.

For the administration of substances we will investigate less invasive ways to deliver these other than via a form of injection. For example, for the delivery of glycosphingolipid inhibitors in the cancer models we have previously investigated using the drinking water (with and without sucrose) as well as the addition of the compound to the food however both proved unreliable as a mode of delivery due to the bitter taste. As this compound requires daily delivery over an extended period (4-6 weeks), we run a pilot study to investigate delivery via mini-pump. We have previously used these pumps successfully for the delivery of PTH in mice. For the delivery of compounds via the intra-nasal (IN) route, we will perform pilot studies (3-4 mice) to ascertain any effects on the nasal passage. We will administer saline or glycosphingolipid inhibitor in a volume of 10ul daily for 1 week in the first instance and mice will be monitored daily for signs of irritation (i.e. pawing, sneezing, redness or discharge) prior to performing larger scale experiments.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Using the 3Rs website (www.nc3rs.org.uk) we will consult with the ARRIVE guidelines and other guidelines such as Responsibility in the use of animals in bioscience research.

How will you ensure you continue to use the most refined methods during the lifetime of this project?



We will keep up with 3Rs advances by consultation with the NACWO and NVS, through regular visits to the 3Rs website and by receiving emails and newsletters. I will ensure that any advances or new strategies are communicated and discussed with my team via meetings, emails and provide details of recommended online resources.

Explain the choice of species and the related life stages

Mice are being used to model human disease; how it starts and ways to treat it. Mice will primarily be used as adults other than those mice in the breeding protocol. The aim is to find ways to reduce disease incidence and/or severity.



13. Breeding and Cryopreserving Genetically Altered Mice

Project duration

5 years 0 months

Project purpose

Basic research

Key words

No answer provided

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 is the aim of this project?

This project will use assisted reproduction techniques that are has been designed to provide an efficient service for freezing germ cells (oocytes/embryos/sperm/ovarian tissue) and the recovery of genetically altered strains of mice. The recovery services will extend to rescuing colonies that fail to breed, in vitro allele conversion and re-establishing inbred strains as part of a genetic stability breeding programme. Following recovery, the mice will be transferred to other projects that have been set up to investigate various aspects of mammalian biology.

The majority of the mouse strains will be archived by freezing oocytes, embryos or sperm and following a specific request, embryo transfer techniques, usually in conjunction with IVF will be used to recover live mice.

Occasionally ovarian tissue will be frozen down in order to preserve a unique mouse strain. This will be limited to those occasions when it is not possible to freeze oocytes, embryos or sperm because only a small number of females exist that carry the genetic modification of interest and further breeding is not possible. On these occasions it will only be possible to recover the mouse strain using ovary transfer techniques.

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



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

Why is it important to undertake this work?

By providing a central service we are able to invest in the most up to date equipment/techniques which can be used to freeze/recover mouse strains on behalf of the wider scientific community. By employing highly trained staff who can maintain their proficiency in cryopreservation and recovery techniques we are able to provide an efficient service. In doing so we will help reduce animal numbers and ensure only the most refined procedures are used, thus minimising the animal welfare implications of the type of work we do.

Access to central freezing services enables investigators to preserve the unique REDACTED they have generated for future generations of scientists. This in turn facilitates resources sharing and eliminates the need to remake mouse models that have been frozen down.

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

This project will facilitate sharing unique mouse models of human disease throughout the scientific community, as well as, freezing down their embryos and sperm for future generations of scientists. We will expand and maintain a large archive of mouse strains to safeguard lines for the future. The sharing of quality controlled genetic models should increase the reproducibility of studies as initial mouse stocks will be identical.

Through this sharing process will enable scientific collaborations to be established around the world in the pursuit of better understanding of gene function and the causes of human disease.

We will publish reports in the scientific literature, where we have developed new techniques or a better procedures for exchanging mice or frozen materials. Working on a previous licences we have developed techniques that facilitate the exchange of unfrozen embryos and sperm, as well as, the exchange of frozen sperm on dry ice. Each of these techniques offers a viable alternative to exchanging live mice. In addition, we have improved IVF and sperm freezing procedures making these techniques more efficient and robust which has reduced the number of animals used in these procedures. These improvements have been publish in the scientific literature, presented on open websites e.g. www.infrafrontier.eu and taught on training courses that we host.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

By sharing these unique mouse models we will eliminate the need to recreate the same mouse strain. In doing so, we will eliminate the additional animal and laboratory costs associated with re-making genetically altered mice.

For those REDACTED we have generated, we have applied robust molecular methods to fully validate the modified alleles and to control the background strains. Therefore, we can disseminate validated models and the recipients can be confident in the knowledge that they are working on the correct genetics. For those strains we recruit into the public



archive from other establishments we gather as much of this information as possible and share it with the community.

By sharing gold standard mouse strains from a well managed and securely funded embryo bank we ensure that collaborating scientists work on identical mice. This in turn aids the comparison of results between laboratories and helps to improve the reproducibility of biomedical data.

Researchers around the globe will have access to high quality REDACTED allowing study of diseases and/or furthering biological and medical knowledge.

By archiving REDACTED that are no longer part of ongoing research projects we can reduce the number of mice being bred. We also eliminate the need to recreate mice that can be quickly withdrawn from the archive.

How will you maximise the outputs of your work?

To maximise the impact of what we do, all the mouse strains that we freeze down on behalf of the scientific community will be presented on our institutional website and a public website (www.infrafrontier.eu) managed by the European Mouse Mutant Archive (EMMA). We also promote our services as widely as possible through information leaflets, workshops, presentations and conference proceedings. The EMMA website is also used to present details of the techniques we use so the other institutions can take advantage of our leading practice.

Working in conjunction with colleagues within the EMMA consortium, we also make a significant contribution to a programme of technical development and publish our findings in technical papers and as standard operating procedures on the EMMA website.

Species and numbers of animals expected to be used

- Mice: 118,250

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Some animals used on in this project may be bred in order to establish colonies of animals for further work. Other mice will be injected with hormones to induced superovulation for egg or embryo harvesting. Yet other mice may undergo an operation to allow embryos to be transferred into recipient females who will give birth. These progeny will be given to the investigator who has requested a particular strain of mice.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.



Animals may be group housed or paired, mated and subject to such other non-painful procedures required for conventional breeding. Female mice may be examined for the presence of a copulation plug and culled at a specific time point in embryo development.

There are a variety of possible impacts due to the genetic modifications carried by the mice bred under this licence. Moreover, in a number of cases we will be breeding the genetically altered mice for the first time and we may not be able to predict these effects.

When tissue is required for DNA genotyping, the mildest appropriate method of sampling will be used for obtaining the tissue e.g. ear notching. Rarely due to a technical problem in analysis or the need to genotype at other genetic loci, a second sample may be taken.

Complications due to surgery are rare although wounds may need re-closing. If this is required it will be done under general 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 species)?

The severity of the protocols used under this licence will vary between Mild and Moderate. It is expected that less than 20% of the mice used on this licence will be put on a protocol with a moderate severity limit.

What will happen to the animals at the end of the study?

- Used in other projects

Replacement

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

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

This is a service licence specifically designed to cryopreserve the germ cells (embryos/sperm/ovaries) of genetically altered mouse strains generated by the scientific community so they are preserved for future generations or shared between laboratories without transporting live mice.

What was your strategy for searching for non-animal alternatives?

Because this is a service licence specifically designed to cryopreserve genetically altered mouse strains generated by the scientific community there are no alternatives to the use of mice for this project.

Why were they not suitable?

Because this is a service licence specifically designed to cryopreserve genetically altered mouse strains generated by the scientific community there are no alternatives to the use of mice for this project.

Reduction



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

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

This application is for a licence renewal and the numbers applied for in this licence reflect the number of mice used over the previous 4.5 years along with estimates of the work we anticipate completing during the life time of this project. These figures can only be estimates based on the expected demand for the services provided under this licence. However, due to the changes in the scope of this licence the number are substantially reduced.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We use a limited suite of proven techniques to achieve the objectives of this licence. Our service has been established in order to cryopreserve mouse strains for the long term and ensure we are in a position to distribute the stocks to multiple users. This dictates that we use 4 to 5 males for sperm freezing; 2 to 8 female mice when transferring embryos (depending on the experimental need); 3 to 6 females for an IVF sperm quality control test and 6 to 15 females for an IVF recovery (depending on the experimental need).

Estimated recovery numbers are calculated based on the number of pups needed for the next stage of the project, taking into account Mendelian inheritance.

The procedural work we conduct under this project licence is conducted as a service to other scientific investigators, rather than defined experimental work in its own right and as such it is not appropriate to follow randomisation and blinding protocols.

What other measures apart from good experimental design will you use to minimise numbers?

We ensure we use the minimum number of mice compatible with the work we are doing by ensuring all reagents are vigorously quality control tested and the laboratory staff are highly trained.

We only use the most advanced techniques compatible with our work and continually review our procedures in accordance with developments in the field. In addition, where it is appropriate to do so, we use the embryo recipient females as sentinel animals in our health screening programme rather than breed additional 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.



Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

This project uses the mouse as an experimental model for every aspect it covers. The mouse is used exclusively because this project has been set up to freeze down and store male and female germ cells from animals that carry unique variations in their genes. The mouse is used for this work because it is the lowest order of mammal that can have its genes manipulated with the precision and complexity available using modern molecular biology tools like CRISPR/Cas9.

The methods used in this project are all well established and well understood laboratory techniques. We will inject mice with similar hormones to those used in human IVF clinics to induce superovulation. The eggs we harvest will be used for in vitro fertilisation sessions that have been set up to generate fertile embryos for transfers into recipient females or for freezing down. The embryo transfer techniques we use are, again, very similar to those found in human IVF clinics and are performed to the LASA guidelines for aseptic procedures (LASA 2017 - Guiding Principles for Preparing for and Undertaking Aseptic Surgery). When surgery is performed the operation is conducted under general anaesthesia and pain relief is given via injectable analgesics.

Sperm will also be frozen down for use by future generation of scientists.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The licence has been prepared specifically to freeze down and recover genetically altered mouse strains for future generations of scientists. The mouse strains will have been generated as part of research into the function of genes or the causes of disease. Consequently there is no alternative to using mice for this project.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

This project uses a limited number of well established procedures. Breeding is strictly controlled so we do not produce any more mice than is necessary the work we want to perform.

We use known information to assess the potential welfare costs of the mice that we breed and the welfare of individual mice is followed on daily assessment sheets when it is appropriate i.e. sick animals.

Hormones are administered by injection which is considered to only cause momentary discomfort. To minimise discomfort during injections, we have a single use needle policy which means the needles will remain sharp. To minimise bruising, we also alternate the injection site when giving multiple injections.

We are hoping to develop a technique that will make the hormone preparations more efficient. This will lead to a reduction in the number of mice we need for our work.

We have developed an improved non-surgical embryo transfer technique that can entirely replace the need for abdominal surgery when handling late stage embryos. To



complement this work, we are hoping to improve the embryo culture environment to allow earlier stage embryos to be transferred using the same procedure.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

To ensure our work is conducted to the highest standards and can be reproduced by other scientists we follow, and act on the PREPARE and ARRIVE guidelines. We also have access to statisticians who can advise on the appropriate experimental design and analysis techniques.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will stay informed by reviewing the literature, attending scientific meetings and talking with colleagues in the field.

We have introduced several procedural changes that have been taken up by others in the field e.g. improved sperm freezing and IVF techniques. As keen supporters of the 3Rs we have successfully submitted two grant proposals to the NC3Rs. The first proposal looked at improvements to the standard embryo culture conditions that we hope will improve efficiency and reduce the number of mice needed to generate the embryos we need. The second proposal was designed to improve the efficiency of the superovulation injections we give. Again, the aim was to reduce the number of mice we need for our work.

Explain the choice of species and the related life stages

This is a service licence designed to rederive and freeze down and distribute genetically altered mice generated by the scientific community. As such it is not possible to use any other species than the mouse for this work. To freeze down mouse strains for future generations of scientists it is necessary to harvest embryos and sperm from adult animals so the embryos and sperm we obtain are able to survive the freezing process.





14. Breeding and Maintenance of Gal-deficient Swine

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Pigs	adult

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Animal tissue commonly produces high levels of a carbohydrate antigen called Gal (galactose alpha 1,3 galactose) which is not present in humans. Since humans do not make this carbohydrate they produce anti-Gal antibody, much like occurs in the case of blood group antigens. The research group has shown that Gal is present in commercial porcine and bovine derived biological heart valves (BHVs) and that human antibody binding to Gal accelerates tissue calcification. Antibody induced calcification does not occur using Gal-free tissue from GT-knockout pigs (GTKO). This project is designed to maintain a breeding colony of GTKO pigs to produce tissues for *in vitro* research which aims to establish the basic physical and biological equivalence of GTKO compared to commercial porcine and bovine derived tissue. This will lead to a more fundamental understanding of the process of tissue calcification and the role of antibody in that process. The Gal-deficient GTKO pigs represent a unique resource which forms the core technology needed for the development of Gal-free bioprosthetic devices.

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

Why is it important to undertake this work?

The primary benefit of this PPL is that it provides tissues and materials to researchers developing new therapies for cardiovascular disease. These new technologies are trying to



improve the performance of replacement biological heart valves (BHVs), especially in patients under 60 years of age. This is experimental work but if successful the new valves will broaden the available therapies to treat younger patients, giving them a durable device which will not require lifetime anticoagulation medication and thereby avoids the serious thrombo-embolic risks associated with anticoagulation. This would have a major impact in developing nations with endemic levels of rheumatic fever a major cause of heart valve dysfunction, where the resources to manage patients on anticoagulation are limited and therefore the treatment of young patients may not be optimal or in many cases is not available at all.

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

This project will supply us with GTKO pericardial tissue to support the production of GTKO pericardial biological heart valves for use in ongoing and future *in vivo* studies in juvenile sheep. These studies are designed to compare the biological equivalence of GTKO pericardial biological heart valves to pericardial biological heart valves made with normal (wild-type) tissue. This is necessary to demonstrate to that the mutation in the alpha-galactosyltransferase gene does not compromise the GTKO pericardium in some unexpected way.

This project will also supply tissue for *in vitro* durability testing of our GTKO pericardial biological heart valves and future *in vivo* comparison in juvenile sheep of our GTKO pericardial biological heart valve with commercially available biological heart valves.

If all of these tests are successful, we will be poised to begin producing our GTKO pericardial heart valve under Good Manufacturing Conditions (GMP) with the aim of gaining regulatory approval to start human clinical trials.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

This is an experimental program developing a new GTKO heart valve. If the program is successful, showing physical and biological equivalence of wild type and GTKO tissues/valves, high GTKO valve durability (greater than 200 million cycles) and equal *in vivo* performance compared to existing commercial devices, we will be positioned to begin GMP production of this heart valve which might then be used in a first in man trial.

These valves, because of their lack of the Gal antigen, are expected to resist anti-Gal antibody induced calcification and have improved durability in all age groups but would be most beneficial to younger patients

How will you maximise the outputs of your work?

The results of this work will be published in high quality peer reviewed journals.

Species and numbers of animals expected to be used

- Pigs: 100

Predicted harms



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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Genetically altered pigs with no expected harmful phenotype will be bred under this project. Each animal will be genotyped following collection of DNA by mouth swabbing or blood sampling. Selected pigs will be maintained to maturity for breeding purposes.

All animals are reared to at least 180 day and humanely killed for tissue harvest.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Pigs bred under this protocol are not expected to show any harmful observable difference in appearance, development, and/or behavior due to their genetic modification.

All pigs will be housed and reared and bred as per standard practices for a normal pig colony.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

Mild 100%

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Tissue samples obtained from Gal-deficient GTKO pigs are required for laboratory purposes to understand the impacts of using Gal-deficient porcine materials in bioprosthetic devices and to support development of a new GTKO pericardial biological heart valves.

What was your strategy for searching for non-animal alternatives?

There are no other alternatives.

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?

Animal numbers will be adapted as necessary to satisfy the tissue quantity required for bio-prosthetic heart valve manufacture, and/or biological testing. The estimated animal number is based on the predicted amount of tissue required over the next 5 years.

Typically a breeding colony of 5-6 is maintained, increasing to a maximum of 15 if tissue demand increases.

Animals will only be mated to maintain the health of the line and to satisfy the requirements for provision of basic materials for in vitro analysis.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Animals will only be mated to maintain the health of the breeding stock and to satisfy the requirements for provision of basic materials for in vitro analysis. Care will be taken to ensure the minimum number of animals is produced.

What other measures apart from good experimental design will you use to minimise numbers?

The scale of breeding is matched to the requirements for tissue and maintaining at the minimum breeding stock to maintain the health of the colony (typically 5 animals).

The few excess GTKO animals not used for tissue donation are used to replace breeding stock.

The animals used in this project are used for non-invasive training and teaching purposes and tissues from wild-type animals are available to other groups under our cadaver sharing policy.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Standard porcine husbandry practices are used.



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The material demands for producing replacement heart valves (e.g. the necessary size of the tissue) requires us to use an animal of substantial size. This would exclude smaller mammals, mice or rats, with the GGTA-1 mutation. Swine currently are the source of many biomedical materials and are appropriately sized matched for humans. Bovine strains with GGTA-1 mutations have been reported and bovine pericardium is widely used to make bioprosthetic heart valves. Due to their large body size and small litter size cows require far greater resources and time for genetic modification compared to pig and would likely be a more expensive source of GTKO materials for our work. There is no experience using tissues from non-mammalian sources (birds, fish, or reptiles) for making BHVs and these animals, while less sentient, would likely be wild caught, in limited supply and maybe under protected status.

GTKO tissue is typically sourced from pigs over 180 days old to ensure enough tissue is available to produce the bioprosthetic devices.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animal staff monitor pigs throughout the night at farrowing to ensure any complications are detected early and where necessary assistance provided according to standard veterinary practice.

Freedom farrowing crates, which allow the sow a greater degree of movement during farrowing and prior to weaning are being tested as an alternative to standard farrowing crates. Pigs are weaned at 4 weeks to minimise the time that the sow is restricted in movement, whilst ensuring the welfare of the piglets.

Buccal swabs are collected as the most refined way to genotype the pigs, this is a refinement that has been implemented over the REDACTED to replace tail tipping. Blood sampling remains as a back-up as buccal swabbing larger pigs can be stressful as restraint is more difficult. In the REDACTED only buccal swabbing was actually used.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Home Office Code of Practice
DEFRA Welfare Codes for Pigs

How will you ensure you continue to use the most refined methods during the lifetime of this project?

NC3R website will be used as a source of information of advances in 3Rs, as well as review of the regular updates received from the designated establishment. Any advance considered appropriate in this PPL will be incorporated where possible.

Explain the choice of species and the related life stages



Tissue samples obtained from Gal-deficient GTKO pigs are required for laboratory purposes to understand the impact of using Gal-deficient porcine materials in bio-prosthetic devices and to support development of a new GTKO pericardial biological heart valve.

Tissues are harvested post-mortem from pigs, greater than 180 days of age, to provide sufficient pericardial material for valve manufacture. Adult pigs will be used for breeding purposes to maintain the colony and supply of tissue.





15. Breeding and maintenance of genetically altered or mutant animals

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

To provide and maintain breeding colonies of genetically modified or naturally occurring mutant strains of animals of scientific interest, whose offspring will be used for specific research roles under other project licences or to provide organs/tissues for *ex vivo* research requirements.

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

Why is it important to undertake this work?



The use of genetically modified animals allows for specific gene traits to be studied in a complex physiological environment.

Some naturally occurring mutant strains of animals have incidences of genetic disorders or conditions (e.g. hypertension) for which breeding and maintaining these strains allows their study in a complete physiological environment.

These types of study cannot be achieved by *in vitro* methods alone.

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

The prime output from this licence will be that animals required for both *in vivo* and *in vitro* research projects will have been provided in the most effective and controlled way achievable for the intended requirements, with good control on reduction and refinement in terms of animals bred and used.

In a wider context outputs of better understanding of complex biological/physiological systems would result from the research these animals have been bred for. Publication and open access to research and new information resulting from the breeding of these animals will be available after the end of this period.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

People and animals with the conditions being investigated in research for which these animals are bred, would potentially benefit from better understanding of the respective conditions, from better diagnosis, earlier and better treatment of such disease conditions. Breeding animals under this licence will provide researchers with good quality animals for their research, enabling better reproducibility.

Controlled breeding under this licence will bring 3R's benefits, in reducing the number of animals produced, identify other research uses and reduce potential wastage. The nature of basic research is that some of the animals bred which then transfer to specific research project licences, will not realise full benefits until after a 5 to 10 year timeframe.

How will you maximise the outputs of your work?

Through sharing and making excess animals bred, or the tissues/organs from them, available for other research purposes, maximum use can be made and animal breeding minimised at the Establishment.

Through information dissemination within the Establishment via research management communications, AWERB, NIO communications, and meetings/seminars involving *in vivo* research groups, researchers are informed of animal resources available and encouraged to use existing bred stock wherever this may be possible. Management group at the Establishment discuss all animal requirements with researchers and attempt to supply requirements from already existing resources, to keep animal production to the minimum possible.

Communications between Registered Establishments, communication via AWERB Regional Hubs, allows sharing of any excess capacity from animal breeding, and provide potential to avoid duplication of breeding some genetically altered lines, that can more efficiently be shared between researchers at more than one Establishment.



Species and numbers of animals expected to be used

- Mice: 5750
- Rats: 250

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Under this licence animals will only be bred to maintain the mutant or genetically modified status of each line or strain. Offspring from the breeding will be transferred to other project licences for *in vivo* research or to provide suitable tissues/organs required for *in vitro* research projects.

Some but not all animals may have a small 'ear notch' of tissue removed for purposes of genotyping and/or identification purposes.

Some female rats may have vaginal swabs taken to determine the stage of oestrous, where this is important to know for research use of the animals, whether transfer to another project licence authority, or for Schedule 1 tissue collections.

Some rats may have blood pressure measurements taken using a standard tail cuff (non invasive) method on several occasions each. This may be for selection of future breeding stock or for possible transfer to another project licence authority, or for Schedule 1 tissue collections.

Rats and mice used for breeding will not be maintained after they have reached a maximum age of 15 months or in the case of breeding females will not be allowed to produce more than 6 litters. Animals bred but not transferred for use under another project licence authority will not be allowed to exceed 15 months of age.

All animals will be humanely killed using a schedule 1 method.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The majority of animals to be bred under this licence will expect to be classified as 'sub-threshold' in relation to the adverse effects experienced. Occasionally a strain of animals with a natural mutation or being genetically altered, may develop symptoms of a disease condition, such as heart defect or co-ordination difficulties, as they reach certain ages. Depending upon the condition and experience, such animals are subject to additional health checks by the animal technicians and the Named Veterinary Surgeon (NVS). Should any reach an assessment of 'moderate' severity threshold which may be reasonably expected to progress further, they are humanely euthanised.



Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

Based on previous experience and knowledge of the strains used in these areas of research, more than 97% of all animals to be bred under this licence would be expected to have a severity classification of either sub-threshold or mild. Less than 3% may reach a classification of moderate at which time they are humanely euthanised, should there be concern that they may become either severe severity threshold or being of prolonged duration.

What will happen to the animals at the end of the study?

- Used in other projects

Replacement

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

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

In order to understand the effects of a modified gene or gene elements, requires subsequent examination of gene activity in a complex physiological environment, which is not fully possible using just *in vitro* techniques. Therefore observations based on live animals or complete organ systems is necessary.

What was your strategy for searching for non-animal alternatives?

For each research project, ethical review always seeks to identify non animal methods. Where these are not possible and whole body systems are required, then it is necessary to purpose breed such animals in a controlled and managed approach, as is the intention and need for this licence.

Why were they not suitable?

Whole body/organ systems are necessary to understand complex gene interactions. Currently to provide these live animal systems, it is necessary to breed the genetically modified and naturally occurring mutant animals.

Reduction

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

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



The estimated numbers are based on experience from previous requirements and knowledge of the various research programmes either currently being undertaken by the Establishment, or are being proposed as future research areas within the Establishment.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The animals are closely monitored to ensure that a genetic line can be perpetuated for ongoing research requirements (continuity of research), such that a genetic line is not 'lost' due to poor breeder selection or other issues that prevent subsequent generations from being produced. This has to be closely balanced with avoiding excessive breeding, which would result in significantly larger numbers of animals which may not otherwise be required for research programmes.

For each individual line maintained for a researcher, a 'colony directive' is developed, and regularly assessed by the NACWO with each researcher, to ensure breeding numbers are minimised and periods for which additional breeding to produce research stock are planned for, with specific numbers to be bred.

If a line is known to be no longer required for a period of greater than 12 months, and if not already 'cryopreserved', this option will be investigated. Alternatively if same line is available at another Establishment, the line will no longer be bred at this Establishment but re-acquired if needed in the future from such sources.

What other measures apart from good experimental design will you use to minimise numbers?

Having animal breeding under the control of this 'service breeding licence' and managed by animal technicians within the establishment, is an excellent way of achieving these aims. Consultation with researchers is an ongoing process, to ascertain numbers of animals required at specific time periods. Breeding directives (detailed breeding colony management documents) are established and routinely monitored to ensure the above situation is maintained.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Genetically modified or naturally occurring mutant lines of mice and rats are proposed as the species to be bred under this licence and which will be used within a range of research projects aimed at understanding various medical conditions. These animals will either be transferred to the jurisdiction of other specific licences for *in vivo* projects, or will be used for tissue and organ supply for *in vitro* projects, following humane euthanasia and tissue/organ collection.



Best husbandry and breeding management along with regular observation by trained animal technicians will ensure pain, suffering or any potential for lasting harm are avoided or minimised, due to strict standard operating procedures.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Because many areas of research require whole body systems in order to understand the complex physiological interactions and affects of individual and multiple gene complexity.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Consultation and regular communication with the Named Veterinary Surgeon (NVS) for the Establishment will help identify if new and better treatments are available and which may be of relevance.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

PREPARE and ARRIVE guidelines are utilised for research projects that animals bred under this licence would be used for, as are LASA guidelines, IAT and RSPCA recommendations/advice for housing and husbandry.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Membership of organisations such as Laboratory Animal Science Association, Institute of Animal Technology, attendance at related meetings and conferences on the use of animals in research. Subscribing to and monitoring the array of resources available from sources such as NC3R's, RSPCA, FRAME, UAR and others.

Implementation of advances will be achieved by submission to and consideration by the Animal Welfare Ethical Review Body (AWERB) at the Establishment, and also from the AWERB Regional hub, that the Establishment is affiliated to.

Explain the choice of species and the related life stages

These animals are being used to support research into a wide range of medical conditions and diseases. By breeding under a 'service licence', many strains and genetic lines of animals required by various research teams, can be expertly bred and maintained by animal technicians at the facility, who ensure control on numbers bred and therefore preventing wastage.





16. Breeding and maintenance of wild type and genetically modified mice for the investigation of G protein coupled receptor function in normal physiology and disease

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Animal types	Life stages
Mice	embryo, neonate, pregnant, 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 is the aim of this project?

Cells in our body communicate with each other by releasing chemical messengers from one cell that then bind to and activate receptor proteins on other cells. One of the largest family of receptor proteins are the G protein coupled receptor (GPCR) family which include well known receptors such as those that bind to adrenaline, serotonin and dopamine. Because these receptors are on the outside of cells and because they are involved in many physiological and disease functions the pharmacological industry have been targeting these receptors with new drugs. In fact GPCRs have been the most successful drug targets known to man. Despite this the full potential of the GPCR family has yet to be realised. The reason for this is that we lack fundamental understanding of the biology of many of these receptors and have little knowledge regarding how to target them in disease. This project licence will enable the breeding and maintenance of wild type and



genetically modified mice as well as the harvesting of tissues and transfer of mice to other project licences for the investigation of the biology and therapeutic potential of GPCRs.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 lack effective drugs for the treatment of many of the world's most devastating diseases. This is exemplified by dementia where the treatments for relieving symptoms, such as memory loss, have limited efficacy whilst there are no drugs that stop or slow the disease. Currently, GPCRs offer a huge potential as targets for drugs for the treatment of many human and animal diseases including dementia.

However in order to realise this promise we require a better understanding of fundamental aspects of GPCR biology, how receptors operate in disease and how best to design drugs with the correct pharmacological properties to treat disease whilst minimising side effects. Hence the breeding and maintenance of genetically modified mice described here will allow for these key studies to be conducted.

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

This licence is for breeding and maintenance. The outcomes described below will be from other projects that will receive animals from this licence .

In the neuroscience project we aim to establish the role of GPCRs in the regulation of neurological responses such as learning and memory, anxiety-like behaviours and locomotion. We also expect to determine the impact of GPCRs particularly the muscarinic receptor family in the symptoms and progression of neurodegenerative disease and schizophrenia.

In the lung respiratory project we expect to determine the role of GPCRs in the regulation of lung function and if targeting GPCRs can change the course and symptoms of inflammatory airway disease

In the gut physiology experiments we expect to determine the role of GPCRs gut physiology and visceral pain.

These discoveries will be disseminated in the following ways;

Peer review literature

Scientific meetings in the form of talks and poster presentations

To the general public in the form of press releases, public seminars and social media

We also expect these discoveries to result in further grant applications and both charitable and government grants.



What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Academic Community - will benefit from a understanding of fundamental biology of GPCRs and the understanding of the best ways to target GPCRs to regulate pathophysiological responses.

Pharmaceutical/drug discovery community – will benefit from the validation of new GPCR targets in human disease and an appreciation of the pharmacological principles that can be applied to drug design.

General public – will benefit from the prospect that new methods will be developed to apply to drug discovery against some of the currently most intractable diseases including Alzheimer’s disease and inflammatory gut disease.

How will you maximise the outputs of your work?

In terms of publications in the scientific literature and presenting in research meetings we are very experienced in these areas with strong relationships with editors of the top journals including Science, Cell and Nature as well as being well connected with organisers of major research meetings. Hence we anticipate that we will continue to have strong outputs through these routes. We have also been developing our outputs via social media with both institutional and personal social media outlets being developed including Twitter and Facebook. Finally we are improving of public outreach with visits to local prisons, schools and presenting to REDACTED.

Species and numbers of animals expected to be used

- Mice: 35,000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically the animals will be mated, litters weaned and off-spring used in subsequent mating. Alternatively, off spring may be used for tissue or transferred to other project licenses. Occasionally, tissue samples may be taken for genotyping.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We are expecting no adverse effects of animals bred and maintained in this project.

We do however have a strain where the receptor that controls salivar secretion (the M3-muscarinic receptor) is deleted (knockout). This strain sometimes finds feeding on normal food (chew) difficult. We therefore in a proportion (~2%) of these animals require to feed



them on a wet mash chow. Also there is small propensity (~2%) of the female M3-muscarinic receptor knockout mice to show a bladder dysfunction which will similarly be monitored.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

All the mice bred under this project will have a mild severity.

What will happen to the animals at the end of the study?

- Kept alive
- Used in other projects
- Killed

Replacement

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

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

There are no available cell culture models to investigate the three primary areas of this project namely; (i) neurological disease, (ii) gut inflammatory disease and (iii) lung inflammatory disease. Although we are using human tissue samples as much as possible we require animal model of these diseases in the background of genetic models expressing mutant forms of our target receptors to investigate the role of GPCRs in the context of normal physiology and disease.

We are employing replacement strategies where possible such as those below;

By conducting biochemical experiments such as proteomic and transcriptomic experiments we are looking for early markers of disease that can give reliable indications of drug efficacy thereby reducing the number of animals and the time they are exposed to disease. This is particularly the case in the neurodegeneration studies but also in our inflammatory models.

We are using human tissue with increasing frequency to reduce the number of mice used.

What was your strategy for searching for non-animal alternatives?

We are using human brain tissue (both normal and disease) obtained from registered human tissue banks for the neuroscience projects described here. We are also working closely with clinicians to obtain human lung tissue (both disease and normal) and human gut tissue. We are therefore making every effort to use human tissue to determine the role of GPCRs in normal physiology and disease as opposed to animal tissue.

Why were they not suitable?

Human tissue is preferable to mouse tissue however it is not possible to employ genetics to validate the receptor targets in human tissue. It is also not possible to trial drugs that target our receptors in humans - rather we can only test the response to our drug



treatment in resected tissue or from post mortem samples. Hence we aim to combine the animal studies with human tissue studies to probe the function of GPCRs in human disease. The ultimate aim will be to subsequently develop drugs based on our findings to trial in human clinical studies.

Reduction

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

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

We have ~19 mouse strains of mice at any one time which on average is maintained at 30 animals each strain per month which over a 5 year period equates to $30 \times 12 \times 5 \sim 35000$ mice

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We are constantly making attempts to reduce the number of animals in the following ways;

We are using well described protocols to reduce pilot studies and animals used in training.

We have established a strong and robust management system to oversee breeding and maintenance to ensure the most efficient use of animals. This management system involves two dedicated animal technicians that maintain records and inspect the animals on a weekly basis. They are also the point of contact for training and maintaining training records for the group. They are also the "eyes in the lab" reporting on the quality of the workers and compliance with the licence.

What other measures apart from good experimental design will you use to minimise numbers?

We have excellent management systems and data-bases in place to ensure efficient breeding and husbandry of the mice.

Where possible we also share tissue amongst users and importantly co-ordinate studies to most efficiently use mice. We also have a large tissue archive which is well indexed and stored.

Refinement

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



Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

GPCR mutant mice

These will be used to directly determine the role of GPCRs in normal physiology and in disease. In particular we have developed mutant receptors that can not be activated by the natural ligands but instead be activated by drugs. In this way we can not only test the role of the target receptors in normal biology but importantly test the action of drugs that work by activating these receptors. Also we have developed GPCR mutant mice where the receptor is restricted in the number of signalling pathways that can be activated. Such receptor mutants can be used to determine the biochemical and signalling pathways used by receptors to mediate clinically important effects and distinguish these from pathways that lead to adverse responses.

Breeding and maintaining these animals will not lead to any pain, suffering or harm.

Neurodegeneration models

We will breed neurodegenerative models including prion over expressing and prion null mice. There is no suffering or harm associated with the breeding and maintenance of these strains. These strains will be used to determine the impact of GPCRs in the progression of disease and disease symptoms and whether targeting these receptors can modify disease.

Reporter mice for reactive oxygen species

There is no suffering or harm associated with the breeding and maintenance of these strains which will be used to monitor mitochondrial function during neurological disease particularly prion disease and whether targeting GPCRs can impact on mitochondrial activity.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Where possible we will use early life stages (e.g. mouse embryos and neonates) to generate neuronal cultures and terminally anaesthetised animals for histology (e.g. perfusion fixation). However the neuroscience projects require models that most closely resemble human physiology and be models that can be genetically manipulated in this case mice are the most appropriate. Also we wish to test the action of receptors and drugs that might lead to new drugs for human use. The receptors and receptor system need therefore to closely relate to humans and therefore mammalian systems such as mice are the most appropriate.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We are always looking for further refinement of procedures. For example we have been undertaking a study to determine biomarkers of neurodegeneration that can be used to establish early on in disease if targeting our receptors of interest impacts on disease before clinical signs appear. We have also been looking towards methods of evaluating



gut inflammation using mild inflammatory models and assessing sensory neuronal activity using terminally anaesthetised animals.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Our primary published source of guidance on 3Rs is via the national centre for replacement, refinement and reduction in animal research (NC3Rs). This organisation publishes regularly on guidance for researchers. The European Medicines Agency also publish excellent practical guidance on 3Rs. We also pay particular attention to the peer reviewed scientific literature for further methods to refine our protocols.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We have regular up-date sessions and training in new approaches run locally and nationally. We also keep abreast of the published literature and share good practice locally. Importantly, we also have expert collaborators that share good practice and we are always looking at new methods to improve our 3Rs.

Explain the choice of species and the related life stages

We are using mice for the following reasons;

Mice are a species where it is possible to change genes in a process often described as genetic engineering. In this way we are able to change specific genes that encode for proteins such as the receptors that are being studied in this project. Hence by genetically engineering mice we can either delete receptor genes or change them so the receptor can be activated by synthetic drugs. This will allow us to probe the function the receptors. Such approaches are not currently possible with other species.

Using embryos and neonates are the optimum life stages to generate primary neuronal cultures using schedule 1 methods





17. Breeding and maintenance of wild type and genetically modified mice to study effects on vascular function

Project duration

0 years 6 months

Project purpose

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

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Cardiovascular diseases, or diseases which indirectly affect the cardiovascular system have a huge impact on our health, cause many people to die prematurely and cost the Health Service a huge amount of money. This licence aims to breed and maintain genetically altered mice and their tissues, which will be used in experiments to study effects of genetic mutations on function of the blood vessels.

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

Why is it important to undertake this work?



The project will increase our understanding of how blood vessels change when animals have certain genetic modifications such as a lack of an enzyme or receptor which may affect how their blood vessels function. This is important as it can sometimes help to identify how and why the function changes and maybe identify ways in which new drugs can be developed to target these processes.

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

This licence will allow us to maintain and breed a colony of genetically modified animals. The colony will provide live mice and their tissues to study the effects of deletion of this particular gene on regulation of blood vessel function. This will, in due course generate significant new data on blood vessel regulation and the importance of the fat which surrounds blood vessels. We intend to publish this data to share our findings and help advance the field.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

We will learn more about how blood vessel function and, more importantly, how they stop functioning properly when they become diseased. We will find out more about how the fat which surrounds our blood vessels can change during diseases such that it becomes damaging rather than protective.

All of these experiments are carried out using genetically modified animals but these can give valuable information about what may happen in humans and by understanding a disease better we can identify targets for drugs or other therapies which can then be developed and tested.

How will you maximise the outputs of your work?

We aim to publish our research in scientific journals so that our findings can be shared with other researchers working in this area. We also aim to present our findings at national and international meetings so that ideas and novel techniques can be shared and the best approach to investigating our area of research adopted. We collaborate with others in the area so that new skills can be learnt and supporting skills can be used to optimise the programme of research and maximise the outcomes.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



The animals on this licence will simply be used for breeding, their genotype being determined by a small sample removed from their earlobe. This does not have any overt adverse effects on the mice.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Breeding of mice should not cause any ill-effects.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

The single protocol on this licence is mild. Very few if any animals are expected to suffer any more than momentary discomfort during ear notching to determine genotype.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 derive much of our preliminary data from experiments which do not use live animals and this helps us to decide on conditions for studies which do use animals. However, in order to investigate how a particular enzyme or receptor or protein affects vascular function, one of the most effective ways is using animals which are genetically modified so that they lack that enzyme, receptor or protein and that requires mice to be bred under licence.

What was your strategy for searching for non-animal alternatives?

We make extensive use of cells from blood vessels grown using a technique known as tissue culture. From these experiments we can derive a huge amount of data which reduces animal numbers and informs the experimental conditions when we do need to use animals. We also make extensive use of historical samples of preserved tissue and tissue fluids/blood which originally came from animals but can be used in multiple experiments.

Why were they not suitable?

They are suitable for certain experiments which form the bulk of our research. However, in certain experiments- such as measuring how an enzyme (such as AMPK) affects blood vessel function, we are able to breed animals which lack this enzyme and investigate how their blood vessels and surrounding tissues are affected.

Reduction



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

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

The numbers are based on past experience of running breeding colonies of mice which are used to produce animals for experimental projects. In the proposed breeding over the 6 month life of this licence, we expect that up to 1000 mice may be bred and their genotype determined experimentally

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Breeding is tightly controlled so that the colony only generates the numbers of animals required. The colony is closely monitored and animals used promptly so that the size is kept within the limits of this licence. The design of experiments which use these animals under non-licenced procedures follow current guidelines and good practice.

What other measures apart from good experimental design will you use to minimise numbers?

Our breeding programme is tightly controlled so that the colony size is monitored and is of a sufficient size to produce the number of animals needed without significant excess. When a member of my research group uses an animal, tissues are shared between multiple users and across research groups so that maximum use is made of that 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

This project involves breeding mice with specific genetic modifications which are of interest to us. Such modification- removing the ability of the animal's genome to produce a specific protein or enzyme allows us to investigate what effect that has on the animal in terms of its cardiovascular system and how it's blood vessels function. When required for use these mice are usually killed via a humane method and their tissue and cardiovascular system investigated.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We are investigating the cardiovascular system and so the best models of cardiovascular disease are in mammals and this is why we use normal and genetically modified mice. It is



important that our basic scientific research has what is called "transferability"- where the results have relevance to the disease or condition in humans. This is difficult to argue when using some less sentient species in the type of research we do.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The experimental models we use are accepted by the scientific community and we have published a great deal of peer-reviewed data reported our findings with them over the last 10-12 years. Through this period we have refined our techniques to make the breeding successful.

In breeding animals it is necessary to determine their genotype and so a small ear notch is taken which may cause no more than momentary discomfort. Breeding animals may also have their diet modified but, again, this does not have any expected adverse effects.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We design and report all our studies according to the ARRIVE guidelines which are widely accepted and endorsed by the scientific community:

<https://www.nc3rs.org.uk/arrive-guidelines>

How will you ensure you continue to use the most refined methods during the lifetime of this project?

There are several sources of information available including the NC3Rs website:
<https://www.nc3rs.org.uk/>

I also regularly correspond with the NACWOs (animal house staff) regarding the best way to maintain our colony of breeding mice and consult with the NVS where any problems arise and to get the most up-to-date information.

Explain the choice of species and the related life stages

For the types of experiments we are performing we need to breed adult animals as their cardiovascular systems are fully developed.

We choose to use mice as this allows us to breed and use mice which are genetically modified (GM), this allows the genome of the animal to be manipulated so that the protein product of a particular gene is not produced (or in some cases the manipulation can be so that more of the protein is produced). This allows us to use animals which are deficient in proteins we are interested in studying- such as an enzyme known as AMPK.





18. Breeding and production of genetically altered mice

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

This project aims to support basic and translational research by providing genetically altered mice for research projects - both the creation of novel genetically altered strains and the maintenance by breeding of established genetically altered strains.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 underpins a range of research projects and has directly enabled many significant and high- impact research findings that have increased our understanding of several human disorders ranging from infertility and cardiovascular disease to rare, debilitating, genetic conditions.



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

This project will underpin a range of scientific studies aimed at understanding genetic diseases such as cardiovascular disease, cystic kidney disease and developmental disorders. Genes implicated in these conditions will be targeted and the relevant tissues and organs studied. In addition to the supply of tissues, animals will be provided for further experimentation in a centrally co-ordinated way to ensure consistency, maximise efficiency and minimise the numbers of animals being used. The scientific insight underpinned by this project will lead to a greater understanding of these diseases and syndromes and help to identify new treatments. This will lead to research publications, greater information for individual patients and patient groups, and provide resources that will be of benefit to the wider scientific community.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

A particular benefit is the maintenance and generation of high quality GA animals using standard protocols to enable researchers at (or moving to) the establishment to continue their research effectively and efficiently. This facilitates international collaborations and interactions that significantly enhance research. Furthermore, having a single licence enables oversight of all breeding and implementation of consistent processes and welfare assessment to minimise the harms to the animals and maintain accurate phenotype information. This licence currently supports scientists working in the fields of cardiovascular disease and childhood kidney disease but due to the nature of this licence, the focus may change depending upon the types of studies being undertaken.

How will you maximise the outputs of your work?

Enabling collaboration is core component of this project. This has been shown to increase overall efficiency and enhance the scientific outputs of research. Furthermore, there is a rigorous selection process during which each individual project is scrutinised to ensure that the most appropriate and refined methods are employed and that the project has the greatest likelihood of achieving the desired goals.

Species and numbers of animals expected to be used

- Mice: 7450

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The majority of mice are bred to maintain the genetic modification. Some mice will be used to produce new genetically altered strains and this will entail some mice undergoing surgery, for example, to generate sterile males (vasectomy) needed in the process, or female mice receiving hormone injections to induce ovulation to provide eggs for carrying out the genetic modification.



Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The vast majority of mice under this licence are not expected to show adverse effects and are only used for breeding to maintain the genetic modification. A proportion of genetically altered mice will show some features of the human diseases they are designed to model, for example, abnormalities of the kidneys, eyes and brain in the case of Joubert syndrome models, however, these are typically only noticeable following microscopic analysis of the affected tissue. A proportion of mice will be used to produce new genetically altered strains and this necessitates (i) hormone injections to induce ovulation and provide eggs for manipulation, (ii) surgery (under anaesthesia) to render males sterile so they can mate and produce "pseudopregnant" female mice that, following surgery (under anaesthesia) to reimplant manipulated eggs, will act as surrogate mothers for the new genetically altered mice.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

It is estimated that up to 80% of mice under this licence will show no adverse effects and be Mild. Only 25% of animals bred under the Moderate protocol are predicted to exhibit any adverse effects, ie, < 10% of the total number of mice being bred.

What will happen to the animals at the end of the study?

- 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 investigation of single and multiple gene effects can be undertaken in a variety of in-vitro and non-mammalian systems and the researchers make use of a variety of these systems, but some studies require the use of live animals, in order to investigate gene effects in an intact mammalian organism. Although genetically altered animals need to be bred for research purposes, many of the specific research manipulations can be carried out in vitro, on tissues obtained after the animals have been humanely killed.

What was your strategy for searching for non-animal alternatives?

Cells cultured in the laboratory and computer-generated models are widely used by the research teams supported by this application to complement animal studies, however, these are not as absolute alternatives as it is not possible to model the effects of a disease gene on the whole body by only using cells in culture. Furthermore, many of the animals bred under this PPL will be humanely killed to generate tissues for laboratory analysis, including the culture of cells and the generation of data to improve computer modelling, without suffering more than mildly themselves.



Why were they not suitable?

Genetically altered mice typically complement experiments carried out on cells cultured in the laboratory and other non-animal approaches, so non-animal alternatives are an important component of medical research. For example, kidney cells can be isolated and cultured from the urine of patients with cystic kidney disease and disease mechanisms/novel treatments can be investigated using these cells but genetically altered mice are essential to then put these findings into the context of a complex organ and the effects on the whole body and provide a most rigorous preclinical model for testing new treatments.

Reduction

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

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

The estimated numbers are based on previous demand for the maintenance of genetically altered mice and the likely increased demand for the creation of new genetic models due to recent technological developments improving the precision with which genetic alterations can be made.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Holding a single project licence to breed these small colonies enables effective control of the numbers bred. We can also ensure that uniformly high standards of animal care and welfare are applied since the staff involved have extensive experience in this field. We also enable effective sharing of some colonies between different research groups, to allow a reduction in the number of animals produced but not utilised for research procedures.

The centralisation of the production of genetically altered mice provides researchers with access to unrivalled expertise in both design and execution and ensures the highest efficiency thus significantly reducing the number of animals that would be required if individual groups were to attempt to produce their own genetically altered mice. Requests from researchers are scrutinised by a panel that provide expert guidance to ensure the experimental design is optimal and can efficiently deliver the project outcomes making use of the minimum number of mice.

Throughout, the principles of the Assessment Framework for Efficient Breeding of Genetically Altered Animals

(https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773553/GAA_Framework_Oct_18.pdf) are adhered to.

What other measures apart from good experimental design will you use to minimise numbers?

Breeding colonies will be monitored carefully to avoid over-production of animals, and animals of specific genotypes that are produced will be used by more than one research



group whenever possible. Breeding colonies that are not required in the short/medium term will be stored as frozen embryos or frozen sperm, to minimise continued production of GA animals. Where specific genotypes are readily available from academic or commercial sources, mice will be acquired for each study, to avoid maintaining a breeding colony.

Furthermore, we actively participate in a number of projects for archiving and sharing of REDACTED and phenotyping information (eg EMMA, MRC Mouse Network) as well as maintaining informal arrangements for sharing lines such as tissue-specific cre lines.

The numbers of animals used on specific projects will be kept to a minimum by requiring those using the animals to provide details of the experimental designs that will be used and adhering to the principles of the Assessment Framework for Efficient Breeding of Genetically Altered Animals

(https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773553/GAA_Framework_Oct_18.pdf).

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Genetically altered mice have proven to be a powerful way of understanding human disease and providing insights not possible from other sources.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Whilst less sentient animals such as zebrafish can provide some insights into basic aspects of vertebrate development, and are used by research teams to complement mouse studies, to understand conditions affecting such tissues and organs as the kidney, immune system, lungs and brain, a mammal is needed. Wherever possible, the earliest stage of development (least sentient) will be used, however, for example, embryonic stages are not suitable for the study of conditions such as Joubert syndrome that affect kidneys due to the fact that the laboratory mouse kidney is not fully developed at birth and, whilst some early evidence of cystic kidney disease can be observed in the mouse at birth, the kidney does not reach a stage of development corresponding to the neonatal human kidney until the mouse is approximately ten days old.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The majority of genetically altered animals bred under the authority of this project have no clinically deleterious phenotype, or can be maintained as heterozygote animals, so that clinically significant adverse effects are not apparent. Homozygote animals with a



deleterious phenotype are produced only when specifically required for the project, or, if produced during a breeding programme, are humanely killed as soon as possible after a welfare problem is identified.

Deleterious effects in some lines develop only after several weeks, and in these instances, breeding programmes are devised to make use of younger animals, that are then humanely killed before the deleterious phenotype is expressed.

When a new GA line is to be imported for maintenance on this licence, details of the anticipated phenotype will be obtained from the supplier and this will inform the initial decisions in relation to breeding and maintenance of the line. When possible, "mouse passport" data that contains more specific husbandry advice will be sought. During establishment of the initial breeding colony, litter size, number successfully weaned, and any specific adverse effects will be documented by regular (daily) examination of the animals. Husbandry modifications (eg use of soft diet, later weaning dates for smaller juveniles, additional bedding etc) will be adopted as required, as outlined in Wells et al, 2006. If the genetic alteration could lead to immunocompromise, husbandry conditions would be modified appropriately, ranging from more stringent barrier conditions through to maintenance in flexible film isolators.

Genotyping will normally be undertaken using tissue removed when ear-notching for identification purposes. When this is not suitable, and tail-biopsy is required, then a specific justification will be submitted to the AWERB before this method is used. If tail biopsy is required, it will be undertaken under brief general anaesthesia (eg with isoflurane) and analgesia (eg meloxicam) administered.

Any pain associated with procedures such as embryo transfer will be minimised by appropriate use of analgesics. All surgical procedures will be undertaken using aseptic technique, by experienced surgeons, so that the incidence of post-operative complications can be minimized.

Furthermore, we constantly strive to improve the general husbandry of our animals, for example recent developments include widespread implementation of non-aversive handling methods to reduce stress, additional in-cage enrichment, the inclusion of "houses" within the home cage providing a more secure environment for breeding animals to make their nests, and improved bedding that we find reduce neonatal losses.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow the guidance provided within the Assessment Framework for the Efficient Breeding of Genetically Altered Animals

https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773553/GAA_Framework_Oct_18.pdf

We regularly check the latest developments at the NC3Rs website and have discussions both within AWERB and with the veterinary team to ensure the work carried out under this PPL is conducted in the most refined way.

How will you ensure you continue to use the most refined methods during the lifetime of this project?



As a member of AWERB, I participate in numerous discussions and briefings concerning advances in the 3Rs and regularly look for updates from the NC3Rs website. In terms of the specific techniques being carried out, along with the veterinary team, we constantly look to refine our approaches, in particular those of a surgical nature and implement best practice based upon the latest NC3Rs findings, for example, the widespread use of non-aversive handling techniques for routine animal husbandry.

Explain the choice of species and the related life stages

These mice are genetically altered for use in research - typically in order to better understand human diseases and seek new treatments.





19. Breeding Genetically Altered rodents and embryo production

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo
Rats	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 is the aim of this project?

To provide Genetically Altered rodents and wild type embryo's to the research community in the establishment.

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

Why is it important to undertake this work?

There are several common use Genetically Altered colonies used in various projects in our establishment and by providing these strains to the various projects we can ensure that the colonies are bred under best practice guidelines. We work within guidelines which are taken from the Genetically Altered breeding initiative from the Home Office in relation to colonies which will be held under this service licence. We regularly review the number of mice to ensure that the demand is met while preventing the overproduction of mice.

Some Genetically Altered colonies will be maintained for the use of tissue collection only. By maintaining the Genetically Altered colonies on the service licence we can ensure that the colonies are bred under best practice guidelines.



The similarities between mouse and human early development enable the Fertility group to use mouse embryos (0.5-2.5 dpc) to carry out feasibility studies and develop techniques before they are applied to the human. For example, the group have been able to test the functional ability of synthetic proteins which have been shown to have an effect on human fertility. This type of infertility affects approximately 1200 couples per year in the UK. The mouse work is a prelude to the work translating to human oocytes.

The use of Genetically Altered embryos will only occur when we are cryopreserving Genetically Altered colonies which are held under this service licence.

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

We will have animals which are good quality in terms of genetics as we will follow best practice. This involves refreshing the background of strains by mating them out to the genetic background every 5-10 generations.

Strains with either mixed or ambiguous background strain information will undergo genetic monitoring to confirm the background substrain. By carrying out genetic monitoring it will give confidence to the reproducibility of the work.

We also use the breeding data which is generated from Genetically Altered colonies maintained under the service licence in colony sizing calculations. Using sizing calculations will help to make sure that the colonies are sized to produce the number of animals required for experiments whilst also not overproducing animals which will be culled as surplus.

Data will allow us to calculate mortality rates of individual Genetically Altered colonies, thus removing the need to refer to genetic background strain data.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The research community will benefit as we will produce good quality animals for their research with accurate breeding and phenotyping data. By producing animals which have good genetic integrity the reproducibility of experiments is improved. By using colony calculations we can reduce the number of animals being produced and potentially wasted.

How will you maximise the outputs of your work?

As a large establishment, there may be duplication of Genetically Altered colonies and by offering to breed and maintain the Genetically Altered colonies under one service licence we can reduce the number of mice which are potentially wasted. The use of the service licence will be disseminated through the establishment welfare meetings. At the meetings, the benefits of using the licence will be discussed e.g. reducing the number of animals wasted, genetic integrity and reproducibility etc.

Within my regular reviews of colonies at the establishment we can specifically target groups where we identify an issue with the breeding and promote the use of the service licence as an alternative.

Species and numbers of animals expected to be used



- Mice: 27,000
- Rats: 1,000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Most animals on the licence will be Bred and Maintained with no welfare issues. Mice will be maintained to a maximum of one-year-old on the breeding and maintenance protocols. Where required animals will be earmarked to obtain a sample for genotyping. The earmarking will generally take place when the animal is around 2 weeks old.

Intraperitoneal injection of hormones 46-48 hours apart to generate either wild type embryos for the fertility group or to generate transgenic embryos for the cryopreservation of lines. The hormone injections are used to increase the number of ova which is obtained from each female. By using this method we are able to use fewer animals to obtain the required number of ova for scientific outputs. The females are then culled by a schedule 1 method to harvest the embryos/oocytes. The cryopreservation of lines is to either remove a non used line from the shelf reducing wastage or to safeguard from a disaster such as fire or flood. Female mice can be left unmated after the hormone injection depending on the researcher's requirement.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

There are many possible adverse effects due to genetic modification of the genome. Information on expected phenotypes will be collected prior to breeding and will be recorded in a colony passport. Any animals exhibiting any unexpected harmful phenotypes will be culled. In the unlikely event of any animal showing any signs of suffering greater than minor and longer than transient or that compromise normal behaviour in any way, the animal will be terminated by schedule 1.

Any animal which displays an unexpected adverse effect on the moderate breeding and maintenance protocol will be moved to a PPL with the relevant authority as soon as identified.

Ear notching should involve only slight and transient pain and no healing problems. If tail tipping is used then the pain will be controlled using a local anaesthetic, and any bleeding will be controlled by local pressure.

Intraperitoneal injections should involve only slight and transient 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 species)?



Breeding and maintenance of genetically altered rodents (mild) mice we expect 80% to be sub-threshold and 20% mild of the 20,000.

Breeding and maintenance of genetically altered rodents (mild) rats we expect 80% to be sub-threshold and 20% mild of the 1,000.

Superovulation mice we expect 100% to be mild of the 7,000

What will happen to the animals at the end of the study?

- Used in other projects
- Killed

Replacement

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

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

Mice and rats are required to supply the needs of end-users Project Licences. In order to fully understand the effects of human genes and their disease-associated mutants, there is no suitable substitute for a mammalian model. The mouse and rat is the model of choice for genetic modification modelling human diseases because of the availability and ease of manipulation of the mouse ES cells. The justification for their use lies with the end-users Project Licence and cell culture assays will be used wherever possible.

Scientists in the REDACTED require live early stage embryos (0.5-2.5 days)

What was your strategy for searching for non-animal alternatives?

The aim of the service licence is to provide end-users with mice and rats for their studies and the consideration of non-animal alternatives will differ depending on the end users' area of research.

Any researcher who wishes to use the service licence will need to provide justification of why they wish to use animals under the service licence and details of any non-animal alternatives they have considered.

On the request to house animals on the service licence the end-users will need to detail which searches and search engines they have used to find non-animal alternatives.

Why were they not suitable?

The aim of the service licence is to provide end users with mice and rats for their studies and will only be done when non animal alternatives have been considered and rejected. The reasons for this will differ between projects but typically the reasons for this are that a whole organism is required for systems biology or behavioural research.

Reduction



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

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

The majority of this licence concerns the breeding of mice and rats. We have extensive experience of calculating animal numbers on a previous licence and we plan to carry on with this practice. We work with end- users to calculate the minimum number required to reach their scientific goals. This involves estimating the final number of animals required and then calculating the number of breeding animals required using existing best practice guidance. This includes ensuring the appropriate age of animals used for breeding, replacement of breeding animals before productivity declines and the use of production efficiency index calculations to size the colony. All colonies on the service licence undergo a three weekly review where the productivity of each breeding pair/trio is assessed and any which fall outside of our guidance are removed.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The size of colonies held on this licence will initially be calculated on the need of the end-users. They will be expected to provide a rationale for the number of animals required to meet their scientific goal and we will calculate the number of breeding animals required using known breeding data.

Genetically Altered colonies held on the service licence will be subject to regular reviews of the breeding performance. The actual breeding performance will be assessed during these regular reviews using data held on a database. It is these data which is used to calculate colony sizes and identify breeding pairs/trios which fall outside of our guidance.

Where usage of a line has fallen to a point where it is no longer possible to breed efficiently, for example, a line being kept on 'tick-over' and not currently being used for experimental work, we will suggest to the users that we will cryopreserve the line. This reduces the number of animals being produced unnecessarily.

What other measures apart from good experimental design will you use to minimise numbers?

The primary purpose of this service licence is the efficient breeding of Genetically Altered animals.

All strains which are maintained on the service licence will be overseen by experienced animal technicians to ensure that the breeding programmes devised for the colonies are followed. This will help to minimise any potential wastage from overbreeding. Where appropriate lines will be cryopreserved to prevent animals from being produced unnecessarily.

This service licence also provides the opportunity to consolidate the breeding of models used by several end users. This would prevent the wasteful maintenance of several smaller separate colonies to provide each of the end-users with these animals. An example of this would be commonly used cre lines.



Animals are promptly sampled for genotyping so that any animals which are of the incorrect genotype are not kept within the colony. End users must demonstrate that the genotyping protocol is validated and robust before a newly generated model is allowed onto the 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The aim of this project is to provide end-users with mice and rats. This will include models relevant to neuroscience (such as the TARDBP^{*}M337V/Ypet Tlbt line which is used as a model of motor neurone disease), cardiology (such as the TMEM16A line which has altered blood vessel function) and other areas of biomedical science (such as the STIM1 line which has altered calcium signalling, important in the immune response). By consolidating the breeding of these lines and monitoring it with dedicated staff we can ensure that breeding and maintenance is done in the most refined manner. This includes efficient breeding practices and the calculations used to size the colonies. We also routinely refresh inbred lines to ensure the genetic integrity of the lines and prevent potentially harmful genetic drift.

Where a line is no longer being actively used this would be cryopreserved which involves superovulation. This is performed using a very established procedure and is considered to be the most refined. In brief, this involves two timed intraperitoneal injections prior to breeding.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The aim of this project is to provide end-users with mice and rats, the justification for which will depend on their individual scientific goals. Typically, the justification for using rodents is that lower-order organisms do not exhibit the required features. For example, an adaptive immune system which is absent from species such as flies. Adult rodents are required for work involving behavioural testing or investigations into ageing diseases. This makes the use of embryos impractical for these scientific areas of interest. Increasingly, rats are being used for more complex cognitive tasks which mice are unable to learn, so there will be instances where end-users use rats over mice or other species. End users will be required to provide justification to use models on this service licence.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

By using efficient breeding and colony management we can reduce the number of animals being produced which are being culled as surplus. By using the efficient colony



management guidelines we have in place we can ensure that breeding animals are retired from breeding by 8 months of age which reduces any potential for breeding related complications.

Where substances are administered by injection the minimum effective dose and the most refined route will be chosen.

When tissue is required for genotyping ear punch is the default position.

Whilst this service licence includes the option for producing animals for cryopreservation by embryo we will always recommend sperm freezing, as the most refined method of cryopreservation, before we agree to carry out embryo cryopreservation.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Home Office efficient breeding of Genetically Altered animals

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We have in place guidelines for breeding colonies which ensure effective breeding of rodent colonies in line with the principles of the 3R's. These are applied to the breeding on the service licence but also distributed through the establishment to other licence holders who maintain breeding colonies. These guidelines will be regularly reviewed based on any advancements made in this area. These advancements will be identified through regular attendance of conferences (LASA, IAT Congress, NC3R's tech symposium, etc.) and comparisons to other large breeding institutions, such as MRC Harwell and Jackson Laboratories. We receive frequent updates from both MRC Harwell and Jackson Laboratories as well as information from the NC3R's, Norecopa and our internal 3R's subcommittee. The 3R's subcommittee collects advancements from across the establishment and disseminates this information. This enables the wider implementation of the advancements made by individual groups.

Explain the choice of species and the related life stages

In order to fully understand the effects of human genes and their disease-associated mutants, there is no suitable substitute for a mammalian model for certain disease models. Due to the complex interactions which take place between different cells, tissues and organs this can only be replicated in a living organism.

Scientists in the Fertility Unit use early-stage mouse embryos (0.5-2.5 dpc) to carry out fertility studies before applying the technique to human embryos. The mouse ova enables them to test

For cryopreservation, we will use females between the age of 3-4 weeks old as it has been shown that females in this age bracket produce a higher yield of ova and a higher percentage of females which are mated produce fertilised ova.



20. Breeding split gene-drive mice spreading female infertility

Project duration

2 years 0 months

Project purpose

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

Key words

No answer provided

Animal types	Life stages
Mice	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 is the aim of this project?

This project aims to test the efficacy of a safeguarded genetic system known as a split gene-drive to spread a genetic alteration through a laboratory-contained mouse population. If successful, gene-drive may find future application as a breeding tool for reducing the cost, time and number of animals required to generate populations of genetically altered research animal. If applied to spread female infertility, gene drive could also find application as a pest management tool.

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

Why is it important to undertake this work?

Genetically altered animals remain an essential tool for biomedical research; however, the cost, time and requirement for a large number of animals to obtain a few individuals of the desired genotype is prohibitive for many complex models of human diseases, such as arthritis and cancer. A cross between mice that are heterozygous for three unlinked genes



must produce 146 offspring for a 90% probability that one will be a homozygous for all three genes. Gene drive has the potential to ensure specific genetic alterations are passed on to offspring and, therefore, could substantially reduce the cost, time and number of animals required to generate populations of animal models harbouring a desired genotype.

Gene drives also have potential as a management tool for control of invasive pest populations. In agriculture and wildlife, invasive pests are well known: rabbits in Australia, REDACTED Is in the UK, and the omnipresent infestation of rodents around the globe. Invasive vertebrate pests impact the environment, economy and society. Current control methods include shooting, poisoning and trapping, which are costly and largely inadequate, and they often lead to unwanted suffering in target and non-target species. A gene drive spreading female infertility may offer a humane, species-specific and cost-effective alternative to current control methods.

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

The outputs of this project will help assess the feasibility of using gene drive as a research breeding tool for efficiently generating study populations of genetically altered animals. This study will also provide preliminary data to inform the debate on gene drive as a tool for managing invasive mammalian pest populations. The findings will assist with technical development of gene drive technology as a breeding tool and a humane alternative to current pest management tools. Dissemination of the project through mainstream media will encourage public and political discussion on genetic pest control approaches.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Gene drive as a research animal breeding tool

The application of gene drive as a research breeding tool could significantly reduce the time, cost and number of animals required for generating genetically altered animals for biomedical research. Gene drive technology has the potential to guarantee the inheritance of one or more specific genetic alterations. In this way, gene drive could substantially reduce the number of animals used, as well as the time and cost of research involving genetically altered animals. Ultimately, gene drive technology could reduce the resources required for scientific research and fast track the development of treatments and cures for disease. If successful, publication and dissemination of our findings could see gene drive technology being widely applied as a research animal breeding tool in the next 3 years.

Gene drive as a pest management tool

Invasive mammalian pests are a global concern that impact the environment, economy and society. Current control methods include shooting, poisoning and trapping, which are costly and largely inadequate, and they often lead to unwanted suffering in target and non-target species. Therefore, the outputs from this project have the potential to benefit environmental conservation efforts, support native predated species, the economy and those within our society that are impacted by mammalian pest, such as farmers and foresters. The outputs also have the potential to reduce suffering in pest species and non-pest species that are affected by currently deployed control methods. The beneficial outputs would likely not be seen for 10 years or more as this technology would require subsequent scientific investigation, including advanced ecological modelling, and a political and public consensus reached before deployment.



How will you maximise the outputs of your work?

This project contributes to collaborative research efforts to assess the feasibility of genetic pest control tools in vertebrate species. The outputs of this work will play a key role in this growing pool of knowledge and many within this collaborative research community are eagerly awaiting the results from this project. We will disseminate outputs through the research community via personal communication, invited talks, workshops, conference presentations and scientific publication. Outputs will be disseminated through mainstream media channels to encourage public and political discussion on the potential use of genetic pest control approaches.

Species and numbers of animals expected to be used

- Mice: 128

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The gene drive mice used in this project will be mated to generate 16 litters of pups. Within those litters, the percentage of offspring that inherit the gene drive will be quantified. Once the female offspring reach sexual maturity, we will check the fertility status in a select number of female offspring that inherited the split gene-drive system.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

This study is expected to have no impact or adverse effects on animal health other than infertility of the female offspring that inherit the split gene-drive system.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

Infertility of some female offspring is expected to be of mild severity. Loss of pregnancy will occur shortly after conception, at the 2-cell embryo stage. We anticipate no other impact from this study on animal health or wellbeing.

What will happen to the animals at the end of the study?

- Killed

Replacement



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

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

Gene drives transmit through sexually reproduction, therefore, the only way to investigate the potential of the safeguarded split gene drives in this project is in sexually mature animals. This project will use the mouse to study the potential of gene drive in mammalian animals.

What was your strategy for searching for non-animal alternatives?

No non-animal systems were considered, as gene drives transmit through sexual reproduction the assessment of these genetic systems is only possible in animal systems.

Why were they not suitable?

Gene drives can't be tested in non-animal alternatives as they transmit through sexual reproduction. At present, there are no non-animal systems for studying the complex biological processes involved in sexual reproduction for the transmission of gene drives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 only previously reported study on gene drive in mice used a different genetic strategy and, therefore, we can not predict the efficiency of the split gene-drive approach in this study based on their findings. The previous study reported up to 5 successful born litters for the gene drive systems tested.

This study aims to generate 8 litters of pups for each of the two gene drive systems tested.

If we estimate an average litter size of 6 pups, we will generate a total of 96 pups whilst screening offspring. To generate these litters, the project will require approximately 8 split gene-drive breeding males (4 of each genetic system) and the 16 wild type female breeding partners. An additional 8 males will be required to confirm the fertile status of the split gene-drive female offspring once they reach sexual maturity. In total, we estimate this study will use 128 mice.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

For a gene drive to be successful as a research breeding tool or pest management tool it will be required to propagate at a rate much greater than 50% (at least 75%). At this rate of transmission, we will be able to see this effect in the selected sample size. If successful in this initial underpowered study, we will consider the value of undertaking an additional study to statistically validate the most efficient of the two split gene-drive systems tested.



What other measures apart from good experimental design will you use to minimise numbers?

We have chosen to perform a small-scale study before considering a larger-scale study that would offer statistical validation of the most efficient strategy. In this way, we are reducing the number of animals being used and whilst gaining valuable data on the biological potential of mammalian gene drive technology, both as breeding tool and pest management tool. By testing two systems at small-scale we will gain insight into the best strategy to further investigate or develop.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

This project will breed and maintain lines of genetically altered mice. Breeding of animals is a natural behaviour and this is anticipated to have no negative impact of animal health or wellbeing. Females may inherit a female infertility trait, which is expected to have minimal impact of animal wellbeing as loss of pregnancy will occur very shortly after conception.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

This project examines the potential of gene drive technology in mammalian pest species. This is the most accessible, genetically sophisticated and cost-effective mammalian model. As gene drives propagate through sexual reproduction it is necessary that sexually mature adult animals are used in this study.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We anticipate minimal animal suffering during this project. Animals are checked regularly (typically daily) and any animal identified to be suffering at a level greater than anticipated will be managed accordingly by experienced staff.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will design and carry out our experiments in accordance with the PREPARE and the ARRIVE guidelines. If required, we will consult the Experimental Design Assistant of the NC3Rs (<https://www.nc3rs.org.uk/experimental-design-assistant-eda>).



How will you ensure you continue to use the most refined methods during the lifetime of this project?

REDACTED. The researchers involved in this project are active participants in 3Rs research and stay informed on the latest advances through scientific publications, social media channels and discussion amongst colleagues and peers. The Institute is a state-of-the-art facility with the expertise to implement advances in the 3Rs as they evolve.

Explain the choice of species and the related life stages

The mouse is the preferred mammalian model for genetic research. Mice are cost effective because they are relatively cheap and easy to care for. The mouse also benefits from a fast generation interval and is small in size, so convenient to house. It is relatively easy to manipulate the mouse genome compared to other mammalian species. As the mouse is the preferred and most widely used mammalian model for genetic research, gene drive is likely to have the greatest impact as a research breeding tool in this mammalian species. For these reasons, the mouse is the species selected to study gene drive technology in this project.

As gene drives transmit through sexual reproduction, the mice used in this project will be sexually mature adults





21. Breeding, Archiving and Rederivation of Genetically Altered or Natural Mutant Animals

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

To provide a demand-matched supply of genetically altered rodents of high health status and defined genetic quality.

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

Why is it important to undertake this work?

Demand for genetically altered animals continues to increase as the knowledge of the genomic sequence becomes increasingly understood. New emergent technologies including genome-editing tools will see an increase in more sophisticated models become available. This PPL will provide a means to breed and maintain these lines to transfer to other licences for experimental purposes. In addition, sperm and embryos may be frozen to keep genetically altered lines for future work.

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



A mouse passport is generated for all mouse strains. This includes information about background, breeding and phenotypic effects.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Researchers and their programmes of work at our establishment.

How will you maximise the outputs of your work?

Animals will be issued as requested and pairs set up to replace the issued animals. If larger numbers were to be requested then the breeding would be increased accordingly. This method is designed to keep stocks to a minimum. Wherever possible standing orders will be encouraged so supply can be accurately controlled and stocks kept to a minimum. All strains entering or leaving the unit are accompanied with a mouse passport which gives in depth detail about the strain and identifies any phenotypic effects the gene modification may have.

Species and numbers of animals expected to be used

- Mice: 7000
- Rats: 700

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The majority of the animals will be used for the breeding and maintenance of genetically altered or non- harmful mutant animals.

Some animals may undergo surgical procedures to either render the animal sterile or to be able to implant embryo's

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Animals bred and maintained under this protocol are not expected to exhibit a phenotype that will exceed mild severity. However, it is difficult to predict the nature and severity of any potential defect and for all types of animals there will be careful monitoring for possible side effects/phenotypes:

Alteration in food/drink intake

Any change in behaviour, social interaction, vocalisations etc.



However, genetic manipulation can occasionally result in unplanned and unexpected effects on mouse physiology and development which may impact on overall life expectancy. The vast majority of genetically altered animals bred and used in this protocol are expected to have a lifetime experience equivalent to that of their wild-type background strains.

Animals that have undergone a surgical procedure may suffer some pain and inflammation at the wound site. Analgesic agents will be administered routinely.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

All of the GAA animals bred and maintained on this PPL will experience only mild or in the majority of cases, less than mild pain, suffering distress or lasting harm.

Animals that have undergone a surgical procedure are not expected to experience pain greater than moderate severity.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 the nature of the licence there are no alternatives other than to use live animals to generate more live animals.

Use of live animals for archiving embryos and subsequently rederiving them is a well tried and tested way of obtaining specific pathogen free animals and securing the colony for the long term.

What was your strategy for searching for non-animal alternatives?

N/A

Why were they not suitable?

N/A

Reduction

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



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

Numbers are based on previous Home Office returns

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Breeding programmes can be tightly controlled in accordance with the needs of the end user. Setting pre-determined targets minimises over production of animals which would otherwise be surplus to requirements.

Good working knowledge of the lines being actively bred allows optimum use of those animals in research programmes allows future requirements to be anticipated and minimises duplicate requests.

What other measures apart from good experimental design will you use to minimise numbers?

The ability to freeze down and transfer embryos will reduce animal use by removing the need to keep redundant colonies. It will also insure against loss of lines and allow rederivation of lines if required.

Rederivation will allow access to animals of high health status minimising experimental variation caused by disease. This will give more consistent results and therefore less animal wastage.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice and Rats will be used under this PPL

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

This PPL is based on researcher requirements. Mature animals are required to produce further animals.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The techniques are established and the limitations and potential problems are known.



The animals will be cared for by dedicated animal technologists who have the expertise and skills required in the breeding of the animals.

Experienced animal technicians are able to assess any welfare problems that may occur at an early stage and are able to determine appropriate end points in consultation with the NACWO and NVS.

The transgenic colonies will be maintained to the same high standards as all other animals in the unit, with health screening to FELASA standards.

No animals with genetic disabilities exceeding mild severity will be bred on this licence.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Home Office

Breeding of Genetically Altered Animals Assessment Framework

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Consultation with a regional NC3R's representative who's role is to inform researchers and technicians of 3R's initiatives and funding opportunities. They also share best practice amongst facilities in the UK and encourage technician and researcher involvement in 3R's activities around the country.

Our local AWERB also has a 3R's sub-committee where the 3R's are discussed and new approaches disseminated to researchers in-house.

Explain the choice of species and the related life stages

Age appropriate animals are required for the project.





22. Cancer Pathways in Metabolic Disease

Project duration

1 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

The aim of this proposal is to finish off projects in which we have been studying cancer-associated pathways (and in particular the Mdm2/p53 pathway) in regulating metabolic disease *in vivo*. This has allowed us to understand how p53 controls diseases such as diabetes and obesity and how metabolic stress can activate the p53 signalling pathway.

Another gene of interest we have been studying is the REDACTED, which is important for cell death as well as fat regulation; and how deletion of this gene manifests global metabolic changes in the context of the whole animal. Cohorts for this project are ongoing.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 successfully delivered on the aims of our previous project licence (12 papers published thus far) but there are one or two outstanding experiments which we will complete in the next 6 months (slightly beyond the expiry date of the current licence). It will



be important to take these experiments to conclusion in order to complete our studies, publish our results and deliver on the large grant which funded these works exploring how the REDACTED pathway contributes to metabolic disorders.

A manuscript outlining the functionality of the REDACTED gene is in preparation and will lead on to a body of work specifically interrogating REDACTED in REDACTED which will be covered by a focused project licence (in preparation). This licence will permit us to maintain these colonies until the new licence is in place.

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

New Information: These studies offer the potential to better understand the signalling pathways and biological processes controlling cellular metabolism and will give insight into how cellular wiring goes awry in these diseases in a physiologically relevant system.

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

Products: There may be therapies which have been developed to treat cancer (eg p53-centred therapeutics) which could be used to treat other metabolic diseases and vice versa where drugs such as metformin used to treat diabetes may be identified as novel cancer treatments. The REDACTED knockout mouse model is the first such murine model of REDACTED and has been disseminated to many labs throughout the world.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The knowledge gained from these studies will be of interest to the scientific community (short term) which will lay foundations for future scientific endeavours which could ultimately lead to new therapeutic approaches (long term) benefiting cancer patients and patients who suffer from metabolic disorders such as diabetes and REDACTED.

How will you maximise the outputs of your work?

We will continue to collaborate with colleagues who are experts in this field and disseminate this work through publication and social media at the earliest opportunity. Many international collaborations have been established through sharing of the REDACTED-KO mouse line.

Species and numbers of animals expected to be used

- Mice: No more than 1000

Predicted harms

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



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Genetically altered animals will be bred together to achieve specific cohorts of interest which will be aged and tissue harvested to ascertain the effects of these genetic alterations in the whole animal. Animals may be put on a special high fat diet to modify their metabolism and test how these specific genetic changes adapt to these metabolic conditions. To better understand these changes we may administer labelling reagents prior to humanely culling animals which aids our analysis of the tissue. Animals will be maximally aged to 12 months of age.

A particular cohort of genetically altered animals will be given a high fat diet to induce metabolic changes and predispose to the development of liver cancer. Animals typically develop liver cancer between 4-6 months of age.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Normal animals placed on high fat diet may gain weight but are healthy otherwise. Males with a REDACTED-KO mutation have a slightly reduced body weight but this does not cause welfare concerns and the mice are otherwise healthy. In a small cohort (<40) we expect animals to develop liver cancer which will result in abdominal distension. Any animal displaying discomfort or abnormal signs will be monitored daily and humanely culled if abdominal distension reduces mobility, or the animal also shows signs of subdued behaviour and/or abnormal breathing.

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

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

Most of the animals on this project will have a subthreshold severity meaning they will show no harm. A small proportion (<20%) will be placed on a high fat diet and/or undergo non-invasive imaging which maximally will be a moderate severity due to repeated anaesthesia. Less than 40 animals will develop liver disease or cancer and will exhibit moderate clinical symptoms.

What will happen to the animals at the end of the study?

- Killed
- Used in other projects

Replacement

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

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

Studying metabolism in the lab has obvious limitations and the true effect on energy metabolism as a result of dietary changes can best be achieved in a living organism. Long



term consequence of altered metabolism (e.g. diabetes) is also best recapitulated in the living animal. Furthermore, tissues are made up of different cell types which can all contribute to cues influencing the reaction to metabolic stresses which is often difficult to recapitulate in non-animal alternatives.

What was your strategy for searching for non-animal alternatives?

Our *in vivo* mouse experiments are an extension of solid *in vitro* observations and only progress using mice when sufficient rationale is obtained based on *in vitro* cell culture, including embryonic stem cells and where possible 3D organoids.

Why were they not suitable?

In vitro models do not allow the overall effects of systemic metabolic disease to be fully modelled in different tissues or the circulating blood system of a physiological animal. In addition the long term effects of sustained metabolic changes (such as fat deposition) is difficult to recapitulate in a tissue culture dish or 3D models as these changes elicit systemic alterations within the whole animal.

Reduction

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

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

Most of the animals will be used in a breeding programme to attain the relevant genetic alterations of interest. We request 800 breeding animals because of the complex genetics in the resulting cohort animals. These cohort animals will be transferred to an end-use protocol for ageing and further assessment (not expected to be more than 120). This will allow us to carry out additional timepoints with a reporter mouse to subsidise a timecourse study that is currently near to completion. Experimental cohorts for one or two key studies have already been generated on a transferring licence, for example we anticipate transferring a cohort of no more than 50 animals to be completed under protocol 3 and 30 animals to protocol 2.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Numbers are calculated based on our own experience, using pilot studies in the first instance, to inform on the numbers we require with advice from our in-house statisticians. We try to scale up experiments and stop recruiting into cohorts when we achieve a statistically significant result.

What other measures apart from good experimental design will you use to minimise numbers?

We use inbred strains of mice which are nearly identical to other in genotype resulting in less variability between animals and allow us to use fewer animals to achieve a statistically significant result. We routinely perform pilot experiments using only a few animals, before



scaling up to the appropriate numbers for a full study. We maximise the breeding programme to generate the most effective breeders to create the genetics of interest.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use widely applied techniques to study metabolism, primarily using changes to diet (eg high fat diet) which requires less handling of animals. Regular monitoring of weight/welfare will allow us to complete studies at the earliest endpoint in which we observe a significant result to prevent unnecessary suffering by extending the study period.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The mouse is a mammal and warm-blooded which shares many features of human metabolism not found in other cold-blooded species such as flies and worms. Furthermore with the ease of manipulating the genetics of the mouse, this makes the mouse the lowest and best model organism to understand the genetic changes observed in metabolic diseases.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

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

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

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

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We continually review our processes and take advice from the Named Veterinary Surgeons, Home Office Inspectors and the NC3Rs website. Our technical staff are very proactive in adopting 3Rs advancements such as non-aversion handling and single-use needles.



Explain the choice of species and the related life stages

Genetically altered animals (GAA) allow us to assess the relevance of a particular signalling pathway in the normal physiological and disease settings using the complete animal. This is particularly relevant when studying metabolic disorders. We are using GAA animals to model how a particular gene alteration associated with a genetic disorder called REDACTED manifests the clinical symptoms in these patients.

Metabolic syndrome is an enormous health burden and is closely linked to the formation of liver cancer. We are also studying the commonly altered p53-MDM2 signalling pathway in a metabolic syndrome that predisposes to liver cancer.





23. Cardiovascular and Metabolic Effects of Uraemia

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

Kidney disease, Heart failure, Hypertension, Diabetes, Stroke

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The increased incidence of artery disease is a significant global health issue. Kidney failure is a major trigger for artery disease and around 50% of patients with kidney disease die of the consequences of artery disease eg: strokes, heart attacks. In patients suffering with both kidney disease and diabetes (25–40% of patients with kidney failure are diabetic), the chance of a cardiovascular caused death is even higher. We believe that this combination of diabetes with kidney failure creates an entirely unique disease scenario requiring treatments distinctive from those given to diabetics or kidney disease patients alone, however much more work needs to be done to further our knowledge of the causes of kidney disease and its pathology if we are to improve its treatment.

Our project has two objectives. Firstly to increase our understanding of kidney disease and its effect on the heart and blood vessels.

Our second aim is to work towards identifying new treatment approaches for these conditions in collaboration with the pharmaceutical industry. We do this on a contractual basis using the same protocols that we use in our own research specifically to test drugs developed for kidney disease.

Much research is being done to develop new drugs for treating kidney disease, diabetes and the associated symptoms eg: high blood pressure, however the fact that the kidney is a major site of drug metabolism means that a diseased kidney may not be able to metabolise many drugs rendering them ineffective. To overcome this, an immense diversity of drugs need to be developed and tested.



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

What are the potential benefits that will derive from this project?

In the long term these investigations are expected to benefit medicine, particularly in the fields of kidney and heart disease and diabetes. The possibility that new disease targets may be identified, for which new drugs could be developed will ultimately provide a major benefit to public health.

Collaboration with the pharmaceutical industry also has benefits in terms of animal welfare. If we did not offer our expertise to industry our clients would have to set up their own animal facilities which would be costly, time consuming, would mean greater animal usage and would involve other groups working through the process of training and model introduction in order to build up the necessary skills. Alternatively they would seek out other research establishments willing to collaborate with them perhaps in places which apply less monitoring of animal well-being. In both these cases the overall result would be greater animal suffering.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

We expect to use a maximum of 5500 mice and 3500 rats over the 5 year course of the licence.

Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

In our research we induce kidney disease in the animals either through surgery on their kidneys or by administering a substance that causes the disease. We then treat the animals with a test agent and monitor its effect against the disease. We compare healthy and diseased animals. Diseased animals begin to display symptoms of kidney failure and these symptoms gradually worsen over the course of the experiment. Although some of our disease models have the potential to make the animals profoundly ill, we limit the time of the duration of the disease so that the experiment is finished before the animals reach that severely ill state. During the course of the experiment we perform tests on the animals that may cause minor distress such as taking blood while the animal is conscious held within a restrainer or echocardiography measurements while the animal is anaesthetised. At the end of the experiments animals are placed under deep anaesthesia so that concluding tests/ measurements can be performed. Animals may be killed under anaesthetic or by other humane methods. Blood/organs are extracted for analysis in the laboratory.



Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

The bulk of our work is carried out using kidney and heart cells grown in our laboratory. These experiments combined with computer based studies enable us to test our ideas regarding disease pathways and possible cures. When we eventually find a reliable promising treatment we carry it forward to be tested in a more complex and therefore realistic disease situation, that is, in an animal with kidney disease. We have to perform these final experiments because the response to a particular therapy observed in a single group of cells growing on a plate may turn out to be very different in a complex living organism where there is the possibility of interaction between many different cell types. Mice and rats share many similarities to humans in terms of body responses to disease so they are our preferred animal choice. However we are aware of recent advancements in our field of interest using lower classed animal species eg: fish larvae, and we intend to explore these alternative possibilities in the future.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Over the last 5 years we have reduced our rat usage by 50% by switching to using mice. This has allowed us to use mice which have been genetically modified. Genetic modifications are changes made to the animal's DNA which remove or upregulate a particular characteristic (protein) in that animal which we think may be causing the disease. When we induce kidney disease in that animal we may find that it is protected from the disease compared to normal animals because of that DNA change. In this way our our experiments are more efficient than when using normal animals resulting in a smaller number of experiments required and less animals needed per experiment resulting in a reduced number of animals overall.

We have also improved our methods of inducing kidney disease in mice by changing from a surgical method to one using a modified diet. This has the advantage of being easier to replicate (as surgery is subject to variability depending on the surgeon). Again this results in fewer animals needed for each experiment and has the added advantage of being much less traumatic to the animals. We still use surgical methods in rats because our diet induced disease is not yet compatible in rats however we are continually developing this method with the intention of using it in rats in future. We have further decreased our animal usage by designing experiments in which (where possible) we can use historical data for the 'sham' (operated on but not treated with drugs) animals.

Where surgical methods are unavoidable we ensure that the surgery is performed by a small number of highly experienced staff which limits variability between individual surgeons again meaning fewer animals per experiment.

We employ the '3rs – Reduction' statistical package when planning our experiments. This package is designed to help researchers achieve the experimental outcomes they want using the minimum numbers of animals.

Refinement



Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Over the course of the last project licence we have increased our usage of genetically modified mice allowing us to target our experiments better, using smaller and fewer experiments and consequently resulting in fewer animals used overall. We have introduced some additional procedures which are designed specifically at refinement. The major change is the replacement of surgery induced kidney disease with diet induced kidney disease in mice. This method is expected to produce similar results but without subjecting animals to the trauma or operator variability that surgery inevitably causes. Although we have already been employing this diet we have further decreased its harmful effects by reducing the amount of adenine in the diet and also by allowing interchange between adenine diet and normal diet during the experimental term. Our intention is to continue refining this method specifically by:

1. Adding other components to the adenine diet to improve its taste making it more palatable with the mice so that they gain weight at the same rate as animals receiving normal diet.
2. Developing the diet for rats so that it too can replace surgery as a means of inducing kidney disease in rats.

Recently (following a 'Refinement' publication by another group) we have begun adding softened food onto the cage floor enabling animals (particularly those recovering from traumatic procedures (eg: surgery to induce kidney disease / streptozotocin injection to induce diabetes) to access the food more easily thus improving recovery. We have also reduced the time animals spend singly housed in urine specimen collection cages from previous 48 hours to a current maximum of 24 hours. In our 'streptozotocin (STZ) induced diabetic nephropathy' protocol, we have reduced overall harm by changing the method of STZ administration from one large dose to 5 consecutive low doses spread over 5 days since small doses of STZ are better tolerated by the animals.

Less evolved species eg: fish, are proving to be more and more effective in kidney and heart studies and our long term intention is to devise renal disease models in fish and by so doing reduce our mouse usage.





24. Cell death, inflammation and cancer in the GI tract

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Animal Types	Life Stages
Mice	adult, aged, juvenile
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 is the aim of this project?

The regulation of cell death and inflammation by both genetic and environmental factors is fundamentally important for the maintenance of homeostasis in the gastrointestinal tract. If these factors are not regulated appropriately, they may contribute to the development of gastrointestinal diseases such as inflammatory bowel disease or cancer and may additionally lead to systemic diseases such as sepsis.

We aim to:

define the regulation of cell death, cell shedding and inflammation in the gastrointestinal tract at the molecular level at baseline and throughout the stages of development of gastrointestinal inflammation, chemotherapy-induced mucositis and gastrointestinal cancer.

investigate how genetic and environmental (eg diet and microbiome) factors contribute to the development of gastrointestinal and systemic disease



identify new therapeutic approaches to treat gastrointestinal inflammation and cancer

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

Why is it important to undertake this work?

The gastrointestinal tract plays a very vital and unique role in that it is exquisitely controlled to regulate the absorption of substances across an epithelial cell layer that is only one cell thick, yet prevents the passage of the intestinal microbiome and mycobiome into the body that can cause systemic inflammation. However, the regulation of homeostasis in the gastrointestinal tract and how this is perturbed during the development of gastrointestinal disease is currently not fully understood. Furthering our knowledge of how genetic and environmental factors contribute towards regulating the gastrointestinal mucosa will identify new molecular targets, for which new pharmaceutical products may be developed and may also lead to the development of new diagnostic tests and techniques for gastrointestinal disease.

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

This project will produce a wide range of data that will provide extra knowledge about how the normal gastrointestinal tract functions and how this is perturbed during several gastrointestinal inflammatory diseases and cancer. We aim to publish these findings in high quality publications in high quality peer reviewed journals. Several are anticipated along the themes of NFkB signalling within the gastrointestinal epithelium, mechanisms of chemotherapy and tyrosine kinase-induced gastrointestinal toxicity, intestinal cell shedding mechanisms and their inhibition, genetic and environmental changes to the intestinal barrier (eg by dietary emulsifiers) and intestinal microbiome changes in response to diet or infection. Data will also be presented at national and international conferences such as the Digestive Disease Week conference in the US and the British Society of Gastroenterology meeting in the UK. We aim to contribute to both scientific and societal impact and will engage with public engagement opportunities to present our work to a wider audience locally and nationally.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Short term

Our studies are mainly mechanistic in nature and therefore will benefit the scientific community in the short term. We will identify mechanisms that may then be targeted to prevent gastrointestinal inflammation and cancer. These studies will where possible be informed by human patient data (from literature and locally from clinical trials and strong links with local hospitals) and will also inform the collection of new data from patients.

Long term

We aim to improve patient quality of life by translating our findings into the clinic. Our mechanistic insight will contribute to novel treatment strategies and drug development for IBD and gastrointestinal cancer.



How will you maximise the outputs of your work?

We have numerous collaborations that will help disseminate new knowledge rapidly. Our interactions extend from Universities worldwide to SMEs and large pharmaceutical companies worldwide. We aim to publish unsuccessful approaches alongside our successful approaches in peer reviewed journals.

One particular internal collaboration makes us well placed to try and achieve our long term aims of translating our mechanistic findings into the clinic. We will continue and further our collaboration with the Department of Pharmacology to cross reference how our mechanistic data fits with current understanding of drug interactions, PK/PD modelling, current treatment approaches, clinical trials, human tissue biobanks. Additional support is available from mathematical and computational modellers based worldwide and accessible through a large consortium project with the aim of shortening the time of drug development through clinical trials and reducing the amount of *in vivo* experimentation necessary in drug development.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 50

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

We aim to cause the least suffering possible to animals that allows us to answer appropriate scientific questions about gut health and disease. The diseases and mechanisms under investigation are all associated with the gastrointestinal tract and include inflammatory bowel disease, gastric and colorectal cancer and mechanisms of diarrhoea and cell death in response to various drugs. In order to investigate how to prevent or cure these diseases, we need to conduct animal experimentation, normally in mice but in rare cases, rats may be a more appropriate model organism. Typically, we will either inject a substance or administer a substance in the drinking water or diet and then kill animals to investigate their gastrointestinal tract from 0-1 week. A smaller number of other studies are of longer duration up to 6 months to 1 year (such as during the investigation of *Helicobacter* colonisation or the production of tumours) which takes longer to cause the changes in the gastrointestinal tract. In such long term studies, there is not normally any further intervention during the development of the disease and animals are closely monitored. A small number of surgical studies are used to implant tumours into the intestine as this is the correct anatomical location for the disease we study, in such cases, animals all receive appropriate anaesthesia and pain relief. Other surgical studies are conducted under terminal anaesthetic and mice are not allowed to recover so do not feel any pain or discomfort. At the end of all experiments, animals are humanely killed and tissues are taken for further quantitative analysis in the laboratory.



Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We aim to end some studies before the animals feel any effects of the intervention. This is particularly relevant when we are investigating how the disease is initiated. However, this is not possible in all cases as animals need to develop the disease/condition in order for us to investigate how to prevent or treat it. In such cases, we typically kill animals as soon as they develop the disease and before they have any long term lasting effects. The diseases relevant to these studies are related to the adverse effects of weight loss and diarrhoea and we aim to minimise these effects as much as possible. Animals are monitored and their data recorded on a regular basis and they are killed promptly if they lose more than 20% of their original weight and if they show other signs of ill health such as hunching and reduced movement in order to prevent any further suffering. The vast majority of animals will be killed at the intended experimental end point and show more mild clinical signs of the disease.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

The majority of mice (and potentially a much smaller number of rats) under investigation will experience 'mild' severity during our protocols. Some techniques that require the development of disease will lead to 'moderate' severity banding in these animals.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Whilst standard *in vitro* tissue culture techniques are informative, they are not able to recapitulate the complex environment found in the gastrointestinal tract. The interactions of multiple cell populations such as epithelial cells that form the inside layer of the gut, with cells of the immune system and surrounding stromal cells are needed to address all of our research questions, and interactions such as this are currently unable to be accurately generated in cell culture experiments.

What was your strategy for searching for non-animal alternatives?

We continue to undertake cell death and proliferation assays in cultured cell lines, primary cells and long lived organoid lines (gastroid, enteroid and colonoid) extracted from human and mouse tissue as well as in mice *in vivo*. While cell death assays in isolated cells or cell culture can be informative, they do not precisely reproduce cell death mechanisms *in vivo*. This is because the surrounding environment including neighbouring cells and extracellular matrix, growth factors and circulating hormones profoundly influence cell death, proliferation and inflammation processes. We have made progress in developing co-culture assays between bone marrow-derived dendritic cells and intestinal organoids,



however, immune cells are widely recognised to change phenotype as soon as they are cultured. It is not currently clear as to how many cells are required to mimic the environment in the intestinal mucosa and other cellular compartments such as different immune cells, gut-specific immune cells, myofibroblasts and neurons, blood supply, oxygen supply, currently unknown signalling components are also not considered. Therefore, the complexity of this *in vivo* environment cannot currently be accurately reproduced *in vitro*. *In vitro/ex vivo* experiments will always be performed if possible and will also be used to inform doses and time-points for *in vivo* investigation.

Why were they not suitable?

Whilst the majority of cell lines are cancer derived, the growth and induction of tumours is heavily dependent on the complex environment and cell types only found *in vivo*. For this reason we shall employ either the standard nude mouse model or syngeneic models to study the effect of agents on tumour growth and administration of carcinogens for the induction of carcinogenesis.

Colitis is a complex inflammatory reaction in the intestinal wall involving multiple cell types acting sequentially. This again cannot currently be reproduced *in vitro*. However useful information can be gained by studying biopsies obtained from human patients with colitis. Wherever possible we shall use human biopsy samples in our studies.

Colonisation of the stomach with *Helicobacter species* or distal gut with other bacterial species causes a complex inflammatory and atrophic reaction in the gastrointestinal mucosa. This cannot be reproduced *in vitro*. However, it is possible and valid to study the effects of *Helicobacter species* and other bacterial species on individual signalling pathways in cultured cells. We shall maximise this latter approach as much as possible.

The intestinal microbiome is complex and many bacterial species cannot be cultured *ex vivo*. To address the complexity of the intestinal microbiome, this requires *in vivo* experimentation. Human faecal and urine samples are used in preference, however, it is impossible to control genetics and dietary components in the human population. The use of inbred mice that have the same diet can control for both genetic and environmental effects.

Reduction

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

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

Where possible, we will use tissue culture techniques and human tissue biopsies to address our research questions. We have previously established that a minimum of 4 mice per experimental group are required for reliable data generation in radiation and mucositis studies, 6 mice are required per group for *Helicobacter* infection studies and that there is a greater degree of variation during experimental colitis studies resulting in the requirement for minimum group sizes of 10 mice. We will therefore estimate numbers of animals based on our previous studies and from other published data. When necessary, we will seek statistical advice.



What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The exact number of animals required will vary with each particular experimental design but we will aim to use data from every animal to its full potential. We will first utilise data from our own studies and the literature to inform any new studies. Online tools such as the NC3R's Experimental Design Assistant will then be used to further inform our studies as these will help to build a visual representation of each experimental plan and provide feedback about experimental design, sample size and statistical analysis methods. We will ensure that appropriate statistical power is achieved for each study and will seek statistical advice for complex multifactorial studies. Potential sources of bias will be considered, for instance, the allocation of mice to groups at the start of each experiment will be considered (eg random, weight-matched, sex-matched).

Factors that will determine sample size will include natural animal to animal genetic variability, variability in response to a drug/treatment and the administration route, variations in intestinal microbiota and other environmental conditions within the animal unit. By considering these factors and addressing as best as possible the sources of bias, we will produce reliable and reproducible data from the smallest number of animals possible.

What other measures apart from good experimental design will you use to minimise numbers?

For each and every experiment, as part of good laboratory practice, we will write an experimental protocol which includes:

a statement of the objective(s)

a description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animals/group) and the experimental material

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).

Pilot studies will always be conducted with two mice if we have no prior experience of the experimental approach, time-course or dose and this will be informed by extensive literature searching.

We aim to provide tissues of interest to other researchers interested in different organ systems where possible both at the REDACTED of Liverpool and at other institutions.

I am actively involved with a project (TransQST) that is aiming to reduce the number of necessary *in vivo* studies by replacing them where possible with computational and mathematical models. Models include how intestinal epithelial cells respond to damage inducing stimuli in terms of their dynamics, proliferative activity and their resistance to cell death. Models may inform how many animals are necessary to ensure a reliable result from computational modelling of further doses and time-points that look at the trajectory of disease.



Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The structure and function of the gastrointestinal tract of mice is similar to that of the human and there are many genetically manipulated strains of mice available that are suitable for the proposed studies. Therefore, the majority of our studies will be conducted in mice. We will also occasionally perform experiments in rats particularly to assess whether there are any species differences between rats and mice. Data from our previous and future experiments will be used to determine the minimum doses of substances required to exert biological effects in order to avoid doses that induce significant toxicity. Where possible, time-points will be analysed when there are early signs of disease but no clinical ill health.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Zebra fish and drosophila are being used increasingly for the study of biological systems and these organisms are both suitable for genetic manipulation. The structure of drosophila intestine is however substantially different from that of mammals and would be unsuitable for most of our studies; however, the zebra fish intestine does display structural similarity. Little is currently known about the dynamics of zebra fish intestine, however we will monitor the field of investigation closely and will include zebra fish in our experimental plans when information about the intestinal epithelia becomes sufficient to enable us to design appropriate experiments.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Data from previous and future experiments will be analysed in order to use the minimum doses of substances required to exert biological effects, rather than doses that induce significant toxicity. In addition, where possible and certainly in all initial experiments, we will analyse time-points when there are early signs of disease but no clinical manifestations (e.g. where possible we will analyse development of aberrant crypt foci in the colon rather than actual tumours).

Wherever necessary we shall use local and general anaesthesia to minimise animal suffering. In anaesthetic with recovery protocols, mice will be administered with pain relief. In grafting experiments we shall kill the mice if the tumours grow to more than 12.5mm in diameter. In all long term experiments, animals will be killed if they lose more than 20% of their initial body weight and if they show any other signs of ill health such as hunching, lack of group behaviour or breathing difficulties.



What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow the ARRIVE guidelines as recommended by the NC3Rs

For tumour studies, recommendations by Workman *et al*, British Journal of Cancer, 2010 will be adhered to, for instance to implement a maximal allowable tumour size.

Laboratory Animal Science Association (LASA) best practice guidelines will be adhered to for blood sampling.

The Grimace scale of facial expressions will be used to inform on animal pain condition in mice.

The Experimental Design Hub will be consulted when establishing a new experimental protocol and is available through the NC3Rs website.

There is a wealthy resource of guidance for best practice training and protocols available through the NC3Rs website, for example, handling and restraint, euthanasia, humane endpoints, welfare assessment, microsampling, anaesthesia, analgesia. PIL holders who are required to undertake any of these activities will be signposted to this website.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I and personal licencees will be encouraged to attend all relevant NC3R-related seminars organised by our REDACTED. I encourage all PIL users who operate under authority of this licence to view the 3Rs training video on the NC3Rs website (<https://www.nc3rs.org.uk/contemporary-training-3rs>) and read the documentation on 'Responsibility in the use of animals in bioscience research'. REDACTED to develop organoid co-culture systems to reduce and replace some *in vivo* experimentation. There is a trainers email that is regularly supplied to project licencees and trainers, a newsletter and a local SharePoint site for all animal users that documents any advances in the 3Rs that is maintained by our REDACTED and 3Rs champions. I aim to implement any advice given and will be actively involved with ensuring that all PIL holders are compliant with any recommended advances. We will discuss 3Rs policies and principles regularly at lab meetings and also discuss any advances in this area we have made (particularly in the development of 3D organoid co-culture) with colleagues locally and in posters/oral presentations at meetings.

Explain the choice of species and the related life stages

Mice are the least sentient animal for the proposed studies. All studies have the requirement for a gastrointestinal tract that functions as a human gut functions. Less sentient animals show great differences in how their gut functions compared to humans. The diseases under investigation are normally associated with juvenile to adult and ageing adult human populations and therefore, this is the choice of life stage in our studies.





25. Cell signalling in neural and cardiovascular development and regeneration

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

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 is the aim of this project?

This project seeks to define molecular and cellular mechanisms that help establish functional neuronal and vascular networks and to determine whether these mechanisms contribute to organ regeneration and repair in diseases of the nervous and cardiovascular systems.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 knowledge gained from work carried out under this licence will help us understand the genetic and anatomic basis of birth defects. This knowledge will also provide vital information on pathways relevant for the design of therapeutic strategies aimed at repairing neural and vascular networks in damaged organs.



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

To date, our basic research programme has yielded several seminal contributions to the fields of neuronal and vascular biology that significantly increased the current knowledge base. Thus, we have provided insight into the mechanisms of blood vessel growth and remodelling during normal development, the guidance cues used by neurons and neural crest cells to correctly position themselves in the developing body, and the regulation of blood vessel leakage in the adult organisms. Moreover, we have uncovered roles for some of these pathways in adult nervous system repair.

Our current project will continue to advance knowledge of developmental processes to understand how functional cardiovascular and neural structures form. We will strive for this work to be published in high impact journals with broad readership across fundamental biomedicine and in an open access format. Thereby, this knowledge will benefit national and international researchers in the areas of cardiovascular development, neurodevelopment and molecular genetics.

Much of our work will also have a translational element by advancing knowledge of fundamental pathophysiological processes that are perturbed in human disease. The application of this new knowledge will, in the long term, provide vital new information to achieve clinical goals. For example, our work on signalling pathways and progenitor populations that control the pathophysiology and regeneration of neurovascular tissues may catalyse novel treatments of acquired conditions with vascular or neuronal involvement, such as nerve injury of the limb or cornea. As another example, learning how to grow functional new blood vessels in ischemic diseases or prevent pathological vascular remodelling in eye disease or the asthmatic lung will lay the foundations for therapeutic correction of these processes to alleviate disease severity. To ensure that such outputs are achieved, we will submit suitable findings to journals at the interface of fundamental and translational research that offer an open access format.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

This project will significantly enhance our knowledge of the developmental processes that establish functional cardiovascular and nervous system wiring patterns.

Knowledge of the developmental processes that underlie and promote cardiovascular health and neurodevelopment is key to understanding how we function as adult human beings. An immediate impact of this project is its opportunity to raise awareness and understanding of science and research through public dissemination of novel findings and explaining their significance.

In the medium term, this research will benefit translational researchers aiming to design new clinical tests for inherited diseases and those working on chronic inflammation or in regenerative medicine. For example, our work will identify novel functions for signalling pathways implicated in congenital and acquired disease that may serve as biomarkers and describe novel types or progenitor cells for vascular growth that may be used for preclinical work in the area of organ regeneration.

In the long term, the academic knowledge generated will thereby benefit clinical medicine to impact positively on public health via novel diagnostics and new pharmacological treatments for patients with cardiovascular and neurological disorders.



In summary, the knowledge we will generate will benefit basic and translational scientists, clinicians and commercial sector researchers in the areas of cardiovascular development and disease, neurodevelopment, neural repair and molecular genetics.

How will you maximise the outputs of your work?

To disseminate knowledge widely within the research community, the team will:

Publish data in relevant peer-reviewed international journals, most of them in open access format. They will strive to publish both successful and unsuccessful approaches, as they have done in the past.

Present findings at national and international meetings.

Engage with media teams at our establishment's press office and funding agencies to communicate key findings to the lay public.

Where appropriate, incorporate novel information also into lectures for undergraduate and graduate students enrolled in programmes at our establishment.

To raise awareness and understanding of biomedical research, the team will engage with the public:

To maximise the availability of our research, results from this project will be published in high- impact journals with open access-compliant policies.

The team will promote the findings of our studies on our establishment's web pages.

The team will liaise with our establishment's and the funding agencies' media services to disseminate findings through public websites and national media.

To enable potential clinical exploitation, the team will:

Publish results in a timely manner in journals read by scientists and clinicians and present at national and international conferences attended by scientists and clinicians.

Seek collaborations with clinicians to advance new strategies for translation. Key to achieving this objective is the team's location in an environment of academic - health sciences partnerships between our establishment and NHS hospital trusts, such as, but not restricted to, the REDACTED.

Species and numbers of animals expected to be used

- Mice: 16000

Predicted harms

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



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Most animals in this project will be genotyped and then used as tissue donors after a humane form of killing (Schedule 1 method). A subset of animals will receive injections that do not cause more than mild and transient discomfort. Some animals will be injured non-invasively, for example with a laser or by modulating environmental oxygen, whilst other will undergo superficial or invasive surgery. Some animals will be exposed to mild allergens. Each animal will only undergo one type of surgical procedure only, and only experience this procedure once. Most experiments involving surgery are completed within two weeks.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

A small subset of animals may experience some pain, in particular after surgery. The period of pain is expected to be relatively short for most protocols, i.e. 24 – 48 hours, although it may last longer in the hindlimb and myocardial ischemia protocols. Pain will be controlled with methods advised by the veterinarian. For protocols with a maximum severity classification of mild or moderate, animals will be culled if they lose 10 or 15% of their pre-treatment weight, as this would indicate that pain or other adverse effects stop them from feeding sufficiently. Some animals will receive eye injuries that may impair vision for up to two weeks, but this is not expected to impact on their behaviour, because mice rely heavily on their sense of smell and touch instead of vision. Some animals will receive a leg injury, but this is not expected to impair their movement or feeding behaviour, because mice can move well on three legs; moreover, these injuries typically heal extremely well within 2 weeks. Some neonatal animals will receive a heart injury, but this also heals extremely well within 2 weeks in the majority of cases. Some animals will be exposed to mild allergens, which usually cause only mild symptoms such as sneezing or scratching. Should animals display abnormal behaviour such as a hunched posture, eye scratching autotomy, laboured breathing or lack of movement or weight loss, then they will be killed by an approved method.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

The severity experienced by animals will vary from sub-threshold for the vast majority to mild for 20% and moderate for around 10%. Unexpected phenotypes due to a novel treatment or genotype will likely be rare, as most substances and genotypes to be used have been previously studied. Nevertheless, mice will be monitored closely to ensure that progression to severe clinical signs is prevented.

What will happen to the animals at the end of the study?

- Used in other projects
- Killed

Replacement



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

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

The principal aim of this project is to understand the molecular and cellular mechanisms that control the growth and behaviour of the vascular and neural cells that give rise to the nervous or cardiovascular systems. Specifically, the project seeks to understand the normal and abnormal development of cells in the context of whole organs, and to investigate how such developmental pathways may be exploited to promote organ repair in congenital and acquired diseases. There is currently no experimental approach that can completely replace the use of animals for such whole body fundamental and preclinical studies. To achieve aims of the proposed research, the project will therefore use wild type and genetically altered mice.

What was your strategy for searching for non-animal alternatives?

Experimental studies utilising bioinformatics and tissue culture models have previously identified candidate molecules and cellular pathways that may prevent excessive injury or aid tissue repair and regeneration in vascular or neuronal disease. This project will continue to use such methods to identify further candidates and identify regulatory relationships between molecules and signalling pathways, reducing the number of animals required.

Why were they not suitable?

REDACTED signalling pathways that may control the behaviour of vascular and neural cells in vivo. However, they have not established the precise function of these candidate pathways in vivo. For example, we found that a signalling pathway identified in cultured endothelial cells is not essential for blood vessel growth, as was predicted from in vitro work, but instead controlled neuronal behaviour in vivo. The experiments in this project are therefore designed to test predictions derived from in vitro models in the context of real tissues, which are composed of multiple cell types and in which several different signalling pathways operate in parallel to orchestrate physiological responses.

A second limitation of in vitro work is the inability to provide physiological evidence that a pathway identified in vitro controls the co-patterning of vessels and neurons in vivo, as no suitable in vitro models are presently available to study this interaction properly. For example, blood vessel networks change in response to perfusion and local oxygen demand, but neither parameter can be controlled appropriately in vitro models. Moreover, during organ repair, both neural and vascular networks are influenced by immune cells, which are recruited to sites of injury and release a wide variety of cytokines that can affect the behaviour of vascular and neural cells in ways that are poorly understood and can still not be modelled appropriately in vitro.

The above limitations will be overcome by manipulating candidate pathways through genetic or biochemical means in the context of a living animal and the subsequent analysis of organs with cell biological, histological and biochemical 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?

We will breed approximately 40 different mouse strains with genetic modifications to maintain these modifications or to cross-breed them to each other in novel combinations, and then use the offspring to propagate the colony or for experiments. On average, this breeding programme will generate around 2400 mice per year. Around half of the offspring will be used for further colony maintenance or for experiments. The other half is expected to be of unwanted genotype or sex. Mice of unwanted genotype will be born due to the principles of Mendelian genetics, whereby a carrier trait will only be passed to half of the offspring, and the other half will be wild type. Mice of unwanted sex will be generated, because we need many females for the breeding programme to carry litters and feed their offspring, but we will only need a few stud males carrying specific traits for breeding.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Mouse colonies will be maintained at the minimum size required to generate sufficient animals of appropriate genotypes and to allow repeats of experiments for statistical comparisons.

The breeding scheme used will minimise the generation of harmful mutants and involves mating asymptomatic carriers of harmful genetic modifications to wild type mice to maintain the colony or to each other or to asymptomatic carriers from other stains carrying different mutations. To minimize harm to individual animals, it is therefore unavoidable that we will generate many mice of unwanted genotypes. They will be culled without having undergone any specific procedure or used as controls in experiments to reduce breeding of wildtype animals or import from commercial breeders.

The mouse number used at each age and in each experiment will be kept to the minimum required to achieve reproducible and statistically significant data. Based on past experience, we need at least 5 and at most 10 adult animals or embryos for each genotype, developmental stage or experimental condition to achieve such significance.

Where possible, we will reduce the amount of in vivo work with in vitro models, in particular to identify new candidate pathways, by explanting tissues and generating stem cell lines. The animal number will therefore be kept to the minimum required to achieve the goals of this project.

What other measures apart from good experimental design will you use to minimise numbers?

Where possible, we will perform retinal imaging in longitudinal studies of live animals rather than cohort studies to examine how genetic changes or treatments affect eye health, as this will substantially reduce the number of animals required.

For experiments requiring the production of tissues, we will harvest multiple organs and share them out amongst team members to advance several research projects simultaneously, rather than allowing each team member to produce and use their own 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

To generate information relevant to humans, a mammalian model is important. Mice are the lowest mammalian species suitable for our studies and also provide the most refined model. Firstly, the planned programme of work builds on prior knowledge and experience in ours and many other laboratories on neurovascular development in mice. Secondly, mouse models are available with mutations in the molecules of interest. Thirdly, standardised assays are available that can evaluate vessel growth and neuronal behaviour in mouse tissues *ex vivo* to minimise the animal number used decrease severity.

Caesarean delivery will be used to prevent females from suffering when they develop complications during labour that could harm the safe delivery of their offspring. Caesarean delivery will also be used when there is reason to believe that pups may develop adverse side effects at birth due to congenital defects caused by their genetic make-up.

Oxygen-induced retinopathy and laser-induced choroidal neovascularisation will be used as non-invasive models of human eye disease. The former involves placing pups with their mothers into a high oxygen environment, similar to what is experienced by premature babies and causing similar pathology. The latter model recapitulates neurovascular changes in age-related macular degeneration and is also suitable for longitudinal studies, reducing the number of mice required at different time points compared to conventional methods that involve killing groups of mice at various time points for histological analysis. Despite retinal injury, mice do not show abnormal behaviour, presumably because the retina does not have sensory nerves and because mice do not rely on good vision but mostly on their keen sense of smell.

To investigate human eye diseases with severe visual impairment due to corneal disease, some experiments will need to induce corneal neovascularisation or injure corneal nerves. The former involves placing sutures into the cornea, similar to the procedure performed in humans with corneal transplantation. This procedure is very well tolerated, because the corneal epithelium heals fast, but the ensuing neovascularisation obscures vision. The corneal abrasion model causes moderate discomfort, because epithelial loss exposes sensory nerve endings, similar to the experience of having dust in the eye. However, the injury site heals rapidly in otherwise healthy mice, mostly within 24 hours and almost completely by 48 hours, and eye drops can be used to counter irritation and dry eye sensations.

Some experiments will surgically induce hindlimb ischemia in one of the hindlimbs of adult mice, because it is the most refined models to model key events that occur in human ischemic limb disease. Thus, even though surgical intervention will cause some discomfort, post-operative mice rarely show signs of distress and can walk and reach food



sources without difficulty, because the three other legs remain fully functional. This model causes symptoms akin to those experienced by patients with diabetic limb disease.

Some experimental investigations will require induction of a myocardial infarct via using coronary artery ligation in neonates. These experiments will use neonatal mice rather than adult mice, because they typically recover well, due to the capacity of the neonatal heart to heal muscle and vascular injury. This model is therefore more refined than using adult mice and also more informative to identify the molecular and cellular mechanisms that actively promote repair and may be used to inform treatment of the human condition.

In some experiments, we will induce an asthmatic phenotype via repeated allergen administration. For these experiments, we will administer allergens that only induce mild asthmatic phenotypes and which do not cause anaphylaxis or other serious symptoms in mice. Specifically, we will use a combination of low dose ovalbumin and house dust mite extract, as these allergens are also relevant to a large number of human patients with asthma.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Data obtained from the research in lower vertebrate species, including fish and amphibians, will inform our experiments, but we cannot ourselves rely on these lower species to meet the objectives of this proposal, for the following reasons: Genetic lineage tracing is not currently possible in any species other than mouse. Moreover, many antibodies are available to identify cell types and specific proteins of interest in mouse, rat and human, but are rarely available for lower vertebrate species, including fish and amphibians. Further, the identity and behaviour of hematopoietic cell lineages and immune processes are significantly different between mammalian and lower vertebrate species, but most of our experiments consider hemato-vascular interactions as key processes that regulate physiology and pathology of the cardiovascular system. Amongst the mammalian systems, mice are the lowest mammalian species suitable for the proposed studies and the only species in which we can do genetic lineage tracing. To decrease severity, most mice will be used at the stages that are least sentient. In particular, embryonic forms will be used to investigate developmental defects wherever possible, as this prevents the birth of mice with severe adverse defects due to genetic defects. Neonates will be used instead of juveniles or adults for neovascularisation models when possible, as neonates are considered less sentient. The eye will be used as the preferred model to examine the mechanisms of neovascular disease, because we can cause diseases through non-invasive methods and because mice are not compromised by a temporary impairment of vision.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Where animals are at risk of developing adverse effects due to their genetic status or a procedure, the level of monitoring will be increased over that performed routinely for healthy animals, typically twice or three times daily. Appropriate post-operative care will be provided, including heating pads, and monitor the operated mice until they have recovered fully from anaesthesia. Post-operative pain will be minimised by providing analgesia, as advised by the veterinarian who oversees this project. Mice will be trained by gentle handling prior to carrying out any procedure and returned to their home cages after the procedure.



What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The project will refer to published guidance, including:

<https://www.nc3rs.org.uk/3rs-resources>

https://www.lasa.co.uk/current_publications/

local standard operating procedure approved by AWERB and our veterinarian

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The team working on this project will stay informed about advances in the 3Rs via the NC3R's website, newsletter and local events.

Explain the choice of species and the related life stages

This project will use the mouse as a model organism, because suitable strains of genetically modified mice or natural mutants are already available or can be generated effectively to study the processes under investigation. Moreover, experiments will be able to draw on a large body of existing knowledge for this species to allow rapid progress and a large number of well-established procedures are available to model key aspects of human disease. Importantly, mouse cells have been found to employ signalling pathways and cellular mechanisms similar to human cells to control their behaviour, and it will therefore be possible to study human cells in vitro and mouse cells in vivo in a complementary manner. The project will use all life stages from embryo to adult to understand the origins and impacts of congenital diseases, determine the contribution of the cardiovascular system to the emergence of diseases and investigate neurovascular interactions in development, disease and organ repair.





26. Cellular & Molecular Mechanisms of Skin Development and Repair

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The aim of this work is to understand how tissues repair after injury, with a particular emphasis on the mechanisms of scarring. The majority of our work is on skin, where we are working understand anatomical variations in wound healing since different body sites vary in their mechanisms and quality of repair. We also study tissue repair processes in the ovary, where there is damage inflicted by ovulation, because this wound heals remarkably well and has the potential to inform us about mechanisms of scar-free healing. We hope to ultimately extrapolate our findings from the mouse model to develop new treatments for human wound healing, fibrotic conditions, and site-specific skin diseases.

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

Why is it important to undertake this work?



There are many human skin diseases and wound-associated pathologies with insufficient treatment options. This work tests a novel hypothesis that the development of the skin (which differs depending on the body site) holds clues for the regional variation in wound repair, scarring and disease manifestation. Also, our investigations on tissue repair in the ovary has the potential to help us understand different problems associated with ovulation, including ovarian cancer.

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

This project will lead to new information about: 1) skin development; 2) mechanisms of wound healing in the skin; 3) regional manifestation of skin diseases; and 4) mechanisms of tissue repair in the ovary. This information will be disseminated by publication as well as by conference presentations.

A potential longer-term output may include new treatment approaches to wound-associated pathologies and fibrotic conditions, since this work aims to identify novel therapeutic targets.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Our short-term outputs (publications and conference presentations) will mostly benefit the relevant academic communities. We anticipate that our findings will have significant impact on scientific approaches to studying and modelling skin and wound healing and the reproductive biology field will also gain important information about the ovulatory process.

If our longer-term goal of discovering novel treatment strategies to improve wound repair and limit scarring/fibrosis is realised, those who could benefit would be patients with wound-associated pathologies (from chronic wounds to keloid scars), and a range of skin diseases (e.g. lupus, scleroderma). Our investigations on tissue repair in the ovary has the potential to help us understand different problems associated with ovulation, including ovarian cancer.

How will you maximise the outputs of your work?

The outputs of this work will be maximised namely by wide dissemination of the results (including negative findings) via publication and conference presentations. Moreover, we will continue to collaborate with researchers whose work would benefit from our alternative development biology perspective on tissue repair and associated pathologies.

Species and numbers of animals expected to be used

- Mice: 4500

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



In order to study ovulation in mice, we propose to inject them with hormones to trigger the ovarian cycle. This is a widely-used protocol that does not appear to cause any distress for the mice, as it requires only two injections and simply mimics the natural hormonal cycle of the mice (mild rating). Occasionally these animals may be treated with additional drugs (that are not expected to have adverse effects), or we may use genetically altered mice (causing no obvious health problems) to study the role of specific cells and molecules.

As for the skin wounds, they are usually made to back skin of adult mice, and typically 4mm in diameter (4 may be made on one mouse) or 1cm in length (2 may be made on one mouse) . Occasionally one 8mm wound will be made, or other sites of the body will be wounded to better reflect human diseases. For a small portion of our work, young pups may be wounded since before a certain stage of skin development scarring does not occur and this provides a window of opportunity to better understand the mechanisms of tissue repair. These procedures are performed under anaesthesia with pain relief provided, and are considered of moderate severity.

All animals are humanely culled (Schedule 1) at the end of these experiments.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The mice undergoing procedures on this licence are expected to experience mild or moderate effects, including transient pain and stress due to the skin wounding protocol and treatments by injection. The genetic alteration to mice we will use are not expected to have any (or only mild) ill-effects on 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 species)?

The majority of the animals maintained and undergoing procedures will experience only mild or sub- threshold effects (75%). Only 25% of the mice used on this licence will be subjected to the wound protocol, therefore experiencing a procedure of moderate severity.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

The complexity of tissue repair cannot be fully modelled in vitro or by using computer modelling because of the diverse cell populations and their intricate interactions. For example, recruitment of inflammatory cells from the circulation, or the importance of circulating hormones add layers of complexity that are impossible to fully recreate in culture or in silico systems.



What was your strategy for searching for non-animal alternatives?

We are able to study certain aspects of wound repair ex vivo, with organ/tissue culture. Our team regularly employs culture experiments (using mouse and human tissue) to investigate how specific molecules affect particular aspects of wound repair. We have spent significant time over the last few years developing an in vitro model of extracellular matrix, which mimics the architecture of scar tissue well, and is amenable to manipulation. Ultimately we aim to extrapolate our findings to human wound repair; accordingly, we use human tissue in culture whenever possible.

Why were they not suitable?

It is extremely difficult to have “control” human in vivo wound experiment, or to analyse human ovulation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 maximum number of mice for this work is 4500. This reflects housing up to six genetically altered lines (and the minimum number of animals required for their generation) for approximately 1 year of experimentation each. This will require up to 2500 mice.

For the wound experiments (ovarian and skin) we have performed power calculations based on our experience (6-8 wounds are required per assay per time-point) and have safely estimated the number of mice required to analyse two groups (e.g. control vs treatment/wild-type vs genetically-altered) at numerous time-points. For the skin only, these numbers are then expanded since we will study multiple anatomical sites, at different stages of the life-course. These calculations have led us to predict usage not exceeding 1200 for skin wound studies, and 300 for ovarian wound studies.

Our phenotypic analyses of mouse strains that have novel timing/cell types for their genetic alterations will not exceed 500 mice.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Yes. In designing these experiments we have received statistical advice (in particular for gene expression profiling experiments). Also, as much as possible we use histological approaches since numerous wound parameters can be analysed with only one sample. Finally, we have consulted with a number of NVS over the years to balance the size of the wound/number of wounds per mouse, so that we can have the most statistically powerful data possible, as well as maximising the information gained per mouse, without causing additional distress.

What other measures apart from good experimental design will you use to minimise numbers?



One strategy we use to minimise animal numbers is, experiments are designed with a staged approach so that early in vitro findings rationalise and justify follow-up in vivo work. Also, to keep numbers at a minimum, animals used in the wound studies may also provide the starting material for some of the cell culture experiments. Finally, the numbers of genetically altered mice are minimised by adopting efficient breeding strategies (e.g. keeping homozygous mice when practical).

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use mice to study both ovarian and skin wound healing, with less than 4500 being required over the 5 year project.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The relatively close evolutionary relationship between mouse and human make it an excellent model. Genetic resources in the mouse enable us to manipulate gene expression in different cell types and to genetically label and isolate distinct cell populations. In parallel, we are carrying out studies using human tissue biopsy material that will inform and be informed by, our mouse experiments.

We use the whole animal model in some instances since the complexity of tissue repair including an immune response cannot be accurately modelled in vitro or with computer modelling because of the diverse cell populations and their intricate interactions. Specifically, the proposed research uses a mouse model of wound repair, in which the skin of adult male mice (abdomen, back or cheek) is wounded using a biopsy punch that is 3mm in diameter. These wounds are as small as possible, yet sufficient to elicit a complete wound response and to provide the quantity of tissue required. All wounding procedures are performed under appropriate anaesthesia. During and after all procedures mice will be carefully monitored. In our experience, wounded mice do not show signs of pain or suffering, eating and drinking normally.

In parallel we are also using our animal models to generate source material for in vitro work. The development and use of in vitro methods serves both to reduce the total number of animals used but also offers a refinement to our experimental approaches as it allows us to dissect the influence of individual cell types.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?



We will stay abreast of recommendations in terms of analgesia, and will continue to minimise the wound size/number required for our scientific questions.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Home Office documentation, as well as publications in relevant academic literature will be regularly consulted.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Via the NC3R charity, and through regular communication about best practice with our Biological Services Unit, the NVS and NACWO, and the users group.

Explain the choice of species and the related life stages

The relatively close evolutionary relationship between mouse and human make it an excellent model. Genetic resources in the mouse enable us to manipulate gene expression in different cell types and to genetically label and isolate distinct cell populations. Most of our studies will be conducted in young adults, however occasionally it will be necessary for us to investigate tissue repair in neonates, at a time when the skin is still developing and the cells within the tissue are more flexible in terms of the type of cell that they will become.





27. Characterizing the biological function of select evolutionary conserved REDACTED proteins

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

We aim to learn more about how the oldest members of a family of proteins (named REDACTED proteins, or KZFPs) control which genes are used in different types of cells, and when. We want to understand how they affect how our bodies work and develop, from the beginning of life to adulthood.

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

Why is it important to undertake this work?

REDACTED proteins are the largest group of DNA binding proteins in the human genome - their function is to compact small portions of DNA, making it temporarily inaccessible to most other proteins. KZFPs have a strong preference for repeated segments of our genomes which can copy or cut-and- paste themselves to different or extra places in the DNA. These pieces of DNA are called 'transposable elements'. Many of those are the remnants of viruses which infected our ancestors thousands of generations ago. Scientists agree that KZFPs are responsible for the control of these transposable elements when they are young, and stop them from spreading amongst our genome.



Recent discoveries show that some of the transposable elements which have been in the DNA of humans for a particularly long time may have been domesticated and do useful work for the cell. REDACTED proteins might be still be controlling them, determining when and where they can work, indirectly deciding which genes are in use at any time, and how intensely they are being used. We want to understand more about this and we think we can find out how much and when this matters. We plan to consider the time before birth, and the life of healthy youngsters and adults. We plan to use mice to do this, because in many ways they are surprisingly similar to humans, and we share many of the same REDACTED proteins with them. However, the family of REDACTED proteins is very large, with hundreds of different members, and we plan to focus on the oldest ones as they are more likely to be important.

Actual genes make up about 2% of our DNA, and most scientists study those as they are the source of proteins. We hope that this work we plan to do over the next five years will lead to a better understanding of some of the rest of the genome, and how it helps in controlling the activity of genes. In the past, most scientists thought that transposable elements don't do much, but we have been discovering in the past few years that they play a big role in the life of the cell. We think our work will help doctors understand the medical importance of changes in the DNA of patients when those changes are not in genes. We hope our research will help to understand how transposable elements and KZFPs work together to decide which genes are 'on' or 'off', which we think will prove to be very useful to prevent and treat illnesses.

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

The main outputs from this work will take the form of increased knowledge about the REDACTED protein family, which is the largest family of proteins in our genome and is vastly under characterized. We would better understand their role in the control of transposable elements and its implication in the evolution of gene regulatory networks. We will share these outputs by publications in scientific journals, communications through conferences and the press, and before this, privately discuss preliminary results with other senior colleagues in the field to potentially establish collaborative partnerships.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short term, the outputs will help the scientists in related fields generate new hypotheses to deepen our knowledge of REDACTED proteins (KZFPs), how they participate in the regulation of transposable elements (ancient retroviral sequences in our genome), and their impact on gene regulation. Some high impact publications would affect the larger scientific community, most probably in the form of dispelling some preconceived misconceptions about the noncoding genome.

More importantly, we hope that our outputs will affect the way we analyze data from the rapidly accumulating databases of full human genome sequences, since we are focusing on KZFPs conserved between human and mouse. There, patients have their genome sequenced to try to identify the cause of rare diseases. Right now, the current state of affairs is that most efforts are still focused on genes, representing 2% of the genome. The rest of the mutations are hard to interpret as we have little knowledge of how transposable elements and other regulatory sequences interact – one of the main goals of this project is



to increase our understanding of this study space, and this will undoubtedly help the public in the long term in the form of improved diagnostics.

How will you maximise the outputs of your work?

We will communicate our results in conferences and scientific publications, make sure we publicize major findings in the press, communicate privately with scientists in the field about successes and failures to avoid replicating work unnecessarily and share best approaches and techniques. We plan to publish negative results (absence of phenotype) in open-access journals whenever possible.

Species and numbers of animals expected to be used

- Mice: 3400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures. Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The number of animals required to generate our target genetically modified REDACTED will be kept to a minimum and will experience the minimum harm possible – we will use standardized procedures for superovulation, electroporation of fertilized eggs, and implantation.

On genetically modified animals, no procedures other than observational handling are planned – animals will be observed then sacrificed at predetermined life stages to harvest tissues for RNA sequencing and other genome wide assays. The specific time points will be adapted for specific REDACTED proteins, depending on phenotypes observed. For example, in the case of embryonic lethality, embryos would be harvested and a minimal heterozygote colony would be maintained. At the other end of the spectrum, long term monitoring of adult animals will be performed if we cannot detect an observable phenotype in early life, and they would be sacrificed at a pre-determined time (i.e. 3 months, or 1 year adult life) to obtain tissues for analysis (RNA-seq to quantify changes in gene expression, ChIP-seq to determine what proteins are present on the DNA, ATAC-seq to quantify chromatin accessibility, tissue embedding / observation by microscopy). If a phenotype with any effects on the well-being of the animals is detected, we would perform these analyses on a time point before they manifest to avoid any unnecessary impact on animals.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

As described previously, we have little information about potential phenotypes following the knock-out of specific REDACTED proteins. So far, Zfp568 (a REDACTED protein) has been described as embryonic lethal. Zfp57 and Zfp445 (both evolutionary conserved REDACTED proteins) have been implicated in the maintenance of genomic imprinting, and animals exhibit a small size / weight phenotype. We don't expect dramatic phenotypes



outside of embryonic lethality for most REDACTED proteins we target, even if they are the most evolutionary conserved, but this is largely unpredictable.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

We will be careful in our selection of which targets to study in-depth after initial phenotypes manifest – we will either select life stages where we can study the effects of the deletion of the gene before any adverse effect manifest for the animal, or focus on the targets that yield mild phenotypes. Should one of our targets unexpectedly develop a moderate or severe phenotype, we will carefully consider and weight potential benefits in relation to the adverse effects the animals would experience and seek the required approvals from the Home Office, possibly leading to a request for an amendment to the current project license to perform any work that would necessitate the use of such animals. It is however not our intention neither our goal – ideally our targets will as we predict cause mild or transient phenotypes.

What will happen to the animals at the end of the study?

- 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?

Most planned experiments in our lab will be performed in vitro and are expected to yield a wealth of information about the role played by KRAB-ZFPs in the evolution of gene regulatory networks.

However, there are clear shortcomings to such experimental systems – this includes the limited number of cell types we can keep in culture on the long term, the lack of tissue microenvironment, the inability to observe effects on the overall organization of tissues, organs and complex biological processes (behavior, metabolism and development). Most importantly, the main reason for the use of animals is to monitor for the apparition of phenotypes which might only manifest after a prolonged period of observation (months) or during development, for example very early during embryogenesis. This aspect is critical to demonstrate the domestication of transposable elements in gene regulatory networks, as the participation in a phenotype which benefits the organism fitness is an essential criteria to demonstrate the highest level of function achievable. We envision that in the future the biotechnological development might partially provide ways to avoid using live animals (with for example in vitro artificial tissue / organ culture) but as of now these are not mature enough.

What was your strategy for searching for non-animal alternatives?

We are using a mix of different cellular models in vitro: cancer cell lines, human primary cells, embryonic stem cells that we can differentiate to specific cell types of interest, and



gastruloid systems (in vitro systems that can create embryonic-like structures from stem cells). They are used in parallel with this project to minimize animal usage – however they cannot achieve our goals with the exclusive use of these systems and many aims require the use of animals.

Why were they not suitable?

None of these models can fully recapitulate the developmental process of an organism, and one of our primary aims is to assess the impact of specific evolutionary conserved KZFPs on development. Even differentiation from stem cells to specific cell types is artificial in many ways, with the supplementation of massive doses of cytokines or inhibitors to quickly induce the process – cellular microenvironments and signalling between cell types cannot be reconstituted fully in vitro, and these models are an approximate representation of reality. The gastruloid system generates embryo-like structures, but are somewhat disordered and develop much faster than a real embryo – they also lack important structures, including what would become the head / brain, where many of our targets are highly expressed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 prioritized our list of KZFPs proteins by using multiple parameters and will generate KO mouse models for the top few candidates in search for observable phenotypes. We initially aim to study 5 REDACTED knock-out for specific KZFPs of interest at once, but plan to generate up to 10 REDACTED to find promising candidates. We estimate that each line will necessitate around 300 (initial characterization and achieve all aims) but up to 500 animals (for a possible full long-term phenotypic characterization of 1 or 2 candidates with highly interesting profiles) for its generation and study over the 5-year period covered by this project. Specific experiments that are planned were subjected to a power calculation to ensure that conclusions at predicted thresholds can be made.

Depending on the pattern of expression of specific targets, single or multiple tissues would be tested from the same mouse. Study groups would include wild type animals (littermates) compared with heterozygotes and homozygote deleted for the target. If there is evidence of a sex-specific phenotype (as observed with an imbalance of sexes in pups for example), we would assay age-matched sex- specific groups in parallel. We also plan to assay changes in gene expression patterns potentially induced by loss of a specific gene at various stages of life of the animal (for example, at 3, 6 and 12 months of age).

If the phenotype is related to a block in development leading to embryonic lethality, we would use the same groups and numbers, using embryos at specific developmental stages instead. We plan neither treatments nor experimental perturbation of animals aside from the initial genetic manipulation to knock out a gene of interest. In some cases, we know that multiple KZFPs bind in close proximity on the genome at a subset of their targets, and depending on the viability and fertility of individual mice KO for each KZFP, we will also



assay the viability / phenotype in a double KO mice. Our experimental plan is simple and we will have no problem publishing using the ARRIVE guidelines.

Our plan is to study at least 5 different KZFPs broadly (to achieve our 4 aims) and a few (1-2) different KZFPs in details on a long period. Even though we start with the ones most likely to yield a phenotype and for which we have strong hypothesis, we cannot fully predict the observability of the phenotype. Therefore, we plan for a scenario where up to 10 mice line KO for different targets will need to be generated in total over the 5-year period of this proposal, but we aim for the lowest count possible. For example, we would generate a new line against the next target in our priority list to replace one that is technically difficult to obtain, or to replace one target that unexpectedly yielded a phenotype which results in the animal experiencing moderate to severe negative consequences.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Sample sizes required for transcriptomic techniques we plan to use have been subjected to rigorous power analysis. This has been done with the advice of various individuals from different groups knowledgeable in the matter.

We have selected candidate genes with high likelihood of phenotype based on multiple criteria, including but not limited to patterns of expression and functional activity, underlying enhancer potential of transposable elements they bind, evolutionary conservation, and function of genes near binding sites.

Since we plan to create many mouse models of different KZFPs sequentially, we will also improve the experimental design iteratively should we notice possible ameliorations that were overlooked during the initial preparation phase, and will do so using the best planning tools available, such as the NC3R's Experimental Design Assistant.

What other measures apart from good experimental design will you use to minimise numbers?

We are keeping up-to-date with the latest developments in methods to genetically modify embryos (CRISPR technology) to optimize the way we generate the knock-out REDACTED – for example, switching from microinjection to electroporation of embryos greatly enhanced the survival rate, reducing the number of total animals required to generate any particular knock-out line.

We will use publically available pre-existing REDACTED from appropriate repositories whenever possible – we plan that this will be the case for example if we require reporter cell lines for a specific KZFP we need to study more in detail. We will favor this strategy in this case as knock-in of complex cassettes is still much less efficient than producing a knock-out using CRISPR. However, we find that producing knock-out in-house with the current CRISPR technology has very high success rates, making it a preferable alternative to ES-cell based, non-validated REDACTED that are available for a few KZFPs.

Similarly, we plan our colony management based on the expected litter size of specific genetically altered animals, should it become apparent that they are reduced for any given mouse line deleted for a given KZFP.



We also plan to restrict the number of REDACTED generated / studied to the absolute minimum to achieve our objectives. We will work initially with up to 5 different REDACTED KO for specific KZFPs, but the goal is to reduce this number to a few promising candidates we can study in detail. We have plans to perform initial characterization of up to 10 lines, but this will only be the case if problems arise with the initially selected KZFP candidates. For example, we would discontinue a line should a moderate or severe phenotype unexpectedly manifest, and replace it with another candidate.

We carefully selected our targets based on a wide-array of publicly available datasets encompassing expression, conservation, predicted gene essentiality from CRISPR studies in cell lines, etc.

We also plan ahead and plan to re-use multiple tissues from any particular individual to maximize the data output per animal, and share tissues with other teams in our Department should they need any from sacrificed 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Knockout mice models are the most efficient way to answer questions regarding function of any particular gene, especially those suspected to play roles in development and higher order biological functions. We will use the best standard protocols for the generation of transgenic animals, and continue to update them as new information becomes available. We will be able with the same model to determine multiple objectives at once, such as the determination of the overall gene essentiality for normal development, if it triggers any observable phenotype at the organism level, to be able to quantify changes in gene regulatory networks in particular tissues, and quantify epigenetic changes resulting from the absence of any particular KRAB-ZFP. We don't plan any procedures or protocol that could cause pain, suffering or distress on the genetically modified animals after we obtain them – our plan is simply to observe the animals, sacrifice them at a given time point and study changes at every level possible. If any of our targets unexpectedly causes a phenotype associated with pain, suffering or distress, we will evaluate the animals before apparition of those symptoms whenever possible, and favor other genes of the KZFP family not inducing such effects.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

KRAB-ZFPs appeared in tetrapods (frogs, reptiles, birds and mammals) limiting the species available for in vivo studies to higher organisms. The family is evolving extremely rapidly and is highly divergent between older phylogenetic branches – for example, no KRAB-ZFPs are conserved between amphibians and mammals. The mouse is the only suitable candidate as it shares a significant portion of its KRAB-ZFPs with human (80 conserved out of 350), making it a relevant model for human health in our specific case.



Mouse models have been successfully generated for a few KRAB-ZFPs and conditional KO of TRIM28 and led to interesting phenotypes. Moreover, there is currently biobanks of embryonic stem cells knockout for most genes in mouse, including multiple KRAB-ZFPs we plan to use – we will maximize the use of these resources to avoid generating transgenic REDACTED ourselves, which would also accelerate our plans and diminish the number of mouse required to achieve our goals.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

While we don't know what kind of phenotype could appear, we will monitor animals regularly after generation of a new KO line to assess if any phenotype is visible or quantifiable. Once a phenotype is identified, animals will be continuously monitored to minimize welfare costs on a per-individual basis, as the phenotype might be variable. Our main goal is to prevent any unnecessary suffering or minimize it should it be necessary – we expect our knock-out lines to yield mild or transient phenotypes. Should a moderate or severe phenotype appear unexpectedly, we would stop the use of that line, or perform the planned assays at a time point before the moderate / severe phenotype appears. We will trial the use of non-surgical transfer of CRISPR-treated embryos to decrease the welfare costs on host recipients when we generate the knockout lines.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will stay up to date with publications from the home office, including but not limited to updates to the “Guidance on the Operation of the Animals (Scientific Procedures) Act 1986”, updates on the NC3R website on various topics, including on the refinement and usage of genetically altered mice, as well as the PREPARE and ARRIVE guidelines. We keep up to date with the latest guidelines and best practices for the efficient breeding of genetically altered animals (Such as the Assessment Framework document from the Home Office

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

553/GAA_Framework_Oct_18.pdf). We will keep up to date with publications from LASA (Laboratory Animals Science Association), and the database of information / tips / best practices on both the NC3R website and by our local network.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will liaise regularly with our NACWO, have conversations with other users of the facility including other research groups, and visit various online resources available REDACTED other 3R related online resources such as www.nc3rs.org.uk). We also plan to regularly attend meetings on best practices, and we are monitoring the calendar of such events published on the nc3r website regularly for nearby (UK / Europe) opportunities.

Explain the choice of species and the related life stages

Mice are the most rational choice to study evolutionary conserved REDACTED proteins – this protein family appeared at the dawn of tetrapods (between fish and reptiles / amphibians) and massively expanded in mammals. Although rodents have lost many of the members conserved in most mammals, they still share 80 with humans, which is more



than enough to achieve our goals. While we are using other models, living animals are the only choice to capture potential phenotypes at the organism level.

We purposely didn't choose a specific life stage to leave all options open, as we don't know what will be the effect of deleting specific REDACTED proteins. So far in the literature, work deleting the gene encoding for their cofactor protein TRIM28 shows a range of effects – it is embryonic lethal, but if removed in specific tissues can have phenotypes that develop over months – for example, sex specific cancer in the liver, or a stress phenotype in the brain. While it is unlikely that any single REDACTED protein will have effects comparable with TRIM28, since removal of TRIM28 results in the inactivation of more than a hundred different REDACTED proteins at once, we want to be able to assess effects developing on the long-term, while planning for possible developmental defects. Once we have a better idea of what kind of phenotype manifests for specific targets we will adjust our choice of life stage(s) to study to maximize scientific output while ensuring that animals experience no or minimal discomfort.





28. Consequences of mitochondrial dysfunction in neurological 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
- 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

No answer provided

Animal types	Life stages
Rats	neonate, juvenile, adult, embryo, pregnant, aged
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 is the aim of this project?

The overall aim is to understand the early consequences of energy failure within different nervous system cell types.

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

Why is it important to undertake this work?



By understanding the early consequences of energy failure due to mitochondrial dysfunction in the nervous system the hope is that we will be able to identify drugs for neuroprotection.

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

We will be able to better understand the basis of symptoms as well as the gradual worsening of neurological function in people effected by demyelinating disorders, including multiple sclerosis, where the protective fatty layer called myelin is damaged and lost (demyelination). Understanding why nerve cells die in disorders with loss of myelin (demyelination) is crucial for identifying novel treatment agents and drugs for diseases like multiple sclerosis.

The testing of compounds and drugs to limit neurodegeneration and improve neurological function will lead to testing of such drugs, particularly for repurposing of currently licenced drugs that are used for other human conditions and proven to be safe, in the clinical setting. Screening of compound libraries and approved drugs will further enhance the potential to discover new treatment options for demyelinating disorders.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

People with disorders where the protective fatty layer called myelin is damaged and lost (demyelination), such as multiple sclerosis. Identification of drugs for treating symptoms as well as stopping the gradual worsening of neurological function in patients will be of great benefit in maintaining quality of life.

In the short term, the discoveries made from these studies will improve the understanding of the basis of neurological disability in demyelinating disorders as well as uncover novel therapeutic targets. Further studies will be needed to valid these novel therapeutic targets as well as test compounds and drugs in phase I clinical studies.

How will you maximise the outputs of your work?

We will design experiments with minimal number of animals and avoid reproducing published work, unless absolutely necessary. We have access to a range of expertise to consult in case we need help in the future for delivering the out put.

Species and numbers of animals expected to be used

- Rats: 1000
- Mice: 5000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



Animals will be maintained for 3 weeks from birth and weaned as usual practice. The a small piece of skin, for example from the ear, will be removed so that we can perform a genetic test to identify whether the genes that we are interested in are present or absent in a given animal. The animals will then receive an agent called tamoxifen, a drug that is used frequently treat breast cancer and is safe in animals, using a gavaging method (oral administration). the animals will be maintained according to the recommendations for best practice until a time point where the experiments can be performed. The experiments will including testing behaviour and performance on well established platforms and equipment. Some animals will then undergo a surgical procedure to the nervous system under general anaesthesia where a small lesion will be made in the nervous system. The location of the lesion is such that the animal will not develop a permanent neurological deficit. Animal will receive pain killing agent(s) before surgery and will be closely monitored following surgery for any evidence of discomfort and sickness behaviour. We will study the impact of the lesioning on the nervous system as well as its function. We will administer agents, such as drugs and compounds, to protect the nervous system from the focal lesioning as well as to see if we can improve function such as by preventing fatigue.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Some of the animals will develop performance related fatigue in the absence of a fixed neurological deficit. Eventually this will turn into a fixed neurological deficit and loss of weight. We will focus most of our work on the early stages of the animal and before the fixed deficit becomes evident. However, it will be necessary to maintain some animals until a moderate severity phenotype is evident, particularly when testing the impact of agents such as drugs for its ability to delay and prevent the onset of fixed neurological deficits. Surgery to the nervous system rarely causes a transient weakness in backleg(s) of animals that last for a few hours. We will closely monitor animals for this adverse effect and contact the veterinary surgeons, if it lasts for more than 24 hours and take advice. If an animal fails to improve after 48 hours then we will consider culling it using humane methods and according to guidelines.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

The majority of the animals will have a mild severity phenotype (60-70%). We may maintain animals until a moderate phenotype develops, particularly when testing the impact of agents such as drugs and compounds on their ability to prevent and delay the onset of fixed neurological disability (a maximum of one third of animals that we will use). We will not maintain animals beyond a moderate severity phenotype in any circumstances.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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



Organism based system is essential to test the impact of agents such as drugs and compounds as symptomatic treatment for complaints such as fatigue in patients with neurological disorders. This feature, performance related fatigue, can not be models using non-animal methods tissue. Furthermore, nervous system is a complex organ with many different type of cells work together in order to execute its function. It is extremely challenging to put together the complex interactions in a suitable integrate experimental system to act as an alternative to the use of whole organisms.

What was your strategy for searching for non-animal alternatives?

We have experimental systems based on human inducible pluripotent stem cells (iPSCs) to select drugs and compounds as treatment options for neurological disorders. It is necessary to test the selected agents in a whole organism to understand the establish the impact of agents on neurological function and behaviour.

Why were they not suitable?

The non-animal systems to do mode the complex and integrated networks that exist within the nervous system in the whole organism. Furthermore, it is not possible to test agents such as drugs for symptoms such as fatigue using non-animal systems where such symptoms can not be measured.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 animal numbers are the result of discussion with statisticians and have been derived from power calculations based on published findings and preliminary data. The study design and written protocols will be submitted to the local veterinary surgeons for all experiments to minimize experimental variation and minimise animal numbers. In situations where previous experience is lacking, we will do pilot studies to determine the variance of the primary outcome measure before deciding on the number needed to show statistical significance with a 80% power. We will use the data generated in initial experiments to determine the minimum number of animals needed for each component of the experimental protocols.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We will select the most reliable and reproducible measurement with the least variability as the primary outcome measure whenever possible, particularly when testing the impact of potential treatments. This will decrease the variability of a given set of experiments and decrease the number of animals needed to observe a statistically significant difference (i.e. to adequately power a study). We will use more than one behavioral test (and no more than 4 in a given animal), which helps to decrease the number of animals needed to



achieve a goal and to identify agents such as drugs that can be potential treatments for patients.

What other measures apart from good experimental design will you use to minimise numbers?

We will ensure that the breeding design is such that all or most of the animals can be used for different experiments to minimise unnecessary breeding of animals. We will store sperm rather than maintain certain type of animal line if not needed for experiments. We will share tissue with our collaborators whenever possible rather than attempt to generate and replicate the models in our lab unless it is absolutely essential. Pilot studies will be performed to calculate the minimum number of animals needed for each experiment so that the study can be adequately powered and the use of excess number of animals can be avoided.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Nervous system cell type specific animal models will be used to study the consequences of energy failure within cells such as nerve cells and myelin forming cells. Cell type specific genetic models, using gene knockout methods, causes less harm than non-cell type specific models. Furthermore, most of the experimental planned will be performed when the animals do not show a phenotype or a fixed/permanent neurological deficit. We will study a specific aspect of fatigue, called performance related fatigue, in mice that show energy failure in the nervous system. These animals show fatigue before a fixed neurological deficit becomes apparent. The focus on early and reversible features minimise animal suffering. The lesioning to model the areas of tissue damage in diseases such as multiple sclerosis will be performed in a specific site (focal lesioning). This avoids large parts of the nervous system being damaged and avoids fixed neurological deficits or lasting harm.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Whole organism approach is needed to study aspects of neurological disorders such as fatigue. While it is possible to study such aspects in less sentient species, the loss of myelin (fatty layer that protects the nerve fibres) using methods that are relevant to diseases such as multiple sclerosis requires mice and rats or more sentient species, partly because of the way the immune system reacts when myelin is damaged. It is not possible to assess behavior and performance when animals are anaesthetised.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?



When animals show signs of compromise and sickness behavior we will increase monitoring. All animals are weighed weekly. We will aim to administer agents such as drugs in the least stressful way, by considering dietary administration before gavaging and intraperitoneal injections. Animals will only be trained if needed for the experiment and we will avoid unnecessary training of animals. Analgesia will be routinely administered before surgery, or focal lesioning of the nervous system. To all animals, supportive care will be provided and their environment will be enriched with deep bedding. Mash will be used and animal monitored daily when mice develop a mild deficit and also when the clinical severity becomes moderate. We will use analgesics following all surgical procedures and monitor animals daily and administered when deemed necessary by research, animal house staff or NVS. Any sign of worsening in moderate severity animal or weight loss of >20% will lead to culling and harvesting to tissue. These parameters will be closely monitored from birth to death. Perfusion of animals will be carried out with terminal anaesthesia.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will regularly visit the 3Rs website (<https://www.nc3rs.org.uk/3rs-resources>) and EMA website (<https://www.ema.europa.eu/en/review-update-ema-guidelines-implement-best-practice-regard-3rs-replacement-reduction-refinement>) and take part in local and, when possible, national meeting aimed at 3Rs. We will make data publicly available so that duplication of studies can be minimised internationally.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I will regularly engage with the local veterinary surgeons and colleagues locally as well as nationally. The team will attend 3Rs meeting and submit studies for such meeting. REDACTED. We will continue to implement advances made in 3Rs.

Explain the choice of species and the related life stages

Animal models play a vital part in discovering and testing drugs for neurological diseases where the multiple different types of nervous system cells function in concert. As a result, it is extremely challenging to develop non-animal experimental systems to model the co-ordinated response the nerve cells trigger to a disease state. We have developed animal models where only one particular cell type is effected as this method minimises the impact of the experimental manipulation on the behavior and well being of the animal. As has purposely focussed on the very early stages of the behavioral disturbance, called performance related fatigue. This life stage is when a change in behaviour is apparent after a given physical activity rather than as a permanent feature. By focussing on early life stages we are able to minimise suffering and test drugs and agents at an early stage which will help protect eh nervous system before its too late.





29. COVID 19 vaccine trial

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

No answer provided

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 is the aim of this project?

To test vaccine candidates against SARS-CoV-2 virus, the cause of the COVID 19 pandemic.

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

Why is it important to undertake this work?

The COVID-19 pandemic poses the greatest health threat to mankind since the 1918 Spanish Flu outbreak. The lives of millions of people around the globe are under threat and the financial disruption caused by the measures implemented to limit its spread is endangering the very fabric of modern civilisation. Until vaccines are developed to protect people against the disease it will not be possible to curtail the pandemic.

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

The data generated by the study will show if vaccination with a specially designed synthetic nanoparticle induces an immune response likely to protect against COVID 19. If successful, the study will generate the data needed to support the progression of the



vaccine into trials to determine its safety and confirm its effectiveness. The study will also establish the most effective route of administration for future mouse studies for nanoparticle vaccines.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The prime objective of the outlined work is to develop a vaccine to protect people against COVID-19. If the studies outlined below show that the engineered nanoparticles induce virus neutralising antibodies, and therefore are likely to be effective at preventing COVID-19 infection, then it will be made available to Biotech companies specialising in vaccine development for efficacy and safety testing. If successful, it is expected that the vaccine would initially be made available to health care providers, like the NHS, to protect their staff. Thereafter the vaccine would be used to create herd immunity within the general population to stop the inexorable spread of the virus and the disease it causes.

How will you maximise the outputs of your work?

If the outlined mouse studies are successful, the product will be advanced immediately into the testing phase need to demonstrate safety and efficacy. To this end a network of collaborators combines all the skills and experience required to deliver this project have been established together with contacts within Big Pharma . A sufficient amount of the first nanoparticle to be assessed has already been synthesised to enable testing to progress without delay. It is to be expected that the results of the initial studies will be available within a few weeks of the licence approval. In due course the findings of the study will be published in peer reviewed journals.

Species and numbers of animals expected to be used

- Mice: 280

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Upon arrival in the unit mice will be allowed to acclimatise for a week. A blood sample will be taken from a superficial vessel (not exceeding 50ul) to provide baseline values for subsequent assay systems. The mice will be inoculated with the vaccine by one of four routes, none of which will cause more than mild transient pain. A second blood sample will be taken approximately two weeks after primary inoculation and if analysis of this shows that the mice have produced a good immune response they will be killed by exsanguination under terminal anaesthesia. If analysis of the second blood sample shows an inadequate immune response the mice will be given a second inoculation by the same route. Approximately two weeks after the second inoculation another blood sample will be taken and if analysis of this shows that the mice have produced a good immune response they will be killed by exsanguination under terminal anaesthesia. If analysis shows an



inadequate immune response the mice will be given a third inoculation by the same route and, approximately two weeks later, killed by exsanguination under terminal anaesthesia.

It is to be expected, based on previous published data, that only primary and secondary inoculation will be required for most mice.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The mice are not expected to experience more than mild transient pain at any point during the outlined studies.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

The severity for this study is mild. All of the animals will experience mild transient pain or distress.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Good *in vitro* assays for modelling primary immune responses to vaccination aren't available. This is because of the complexity of the process which involves the uptake and processing of antigen by antigen-presenting cells in tissues, the transport of antigen presenting cells to the lymph nodes and induction of the primary responses at these sites.

What was your strategy for searching for non-animal alternatives?

Non-animal alternatives are not applicable to this study.

Why were they not suitable?

It is not possible to assess the immune response evoked by an antigen *in vitro*.

Reduction

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

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



The initial pilot study will use a group size of 10 animals (see previous section). Four vaccine candidates have been developed and are awaiting testing, a further two are currently planned. During the initial pilot experiment three systemic routes of inoculation will be evaluated plus one mucosal challenge. A separate control group will be used for each of the four inoculation routes used in the pilot study. For subsequent studies, only the most effective systemic route (based on the pilot study data) plus the mucosal route will be used. Control groups will only be included in subsequent studies when there is a clear scientific need, however for the purpose of estimating the total number of animals it has been assumed that they will be required.

Initial Pilot study - (4 vaccinated + 4 control groups) x 10 mice/group = 80 mice

Subsequent studies – (2 vaccinated + 2 control group) x 5 vaccine candidates x 10 mice/group = 200 mice

Total = 280 mice.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The group size was calculated on the basis of published data from previous protein nanoparticle vaccines as set out above.

What other measures apart from good experimental design will you use to minimise numbers?

Follow analysis of data generated by the initial experiment the group size will be recalculated and adjust accordingly.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

This study involves inoculation and sampling methods that cause mild transient pain or distress. None of the animals are expected to experience any adverse effects as a result of the procedures undertaken.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice have the lowest neurophysiological sensitivity of the animals suitable for these studies. It is not possible to conduct this study in less sentient species as they do not have immune systems comparable to humans.



What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The procedure undertaken will cause mild transient pain or distress. Anaesthesia will be used for procedures in which the stress of anaesthesia is less than that endured as a result of performing the procedure.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

All procedures will be conducted in accordance with LASA, NC3Rs Guidelines on best practice for the administration of substance.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I am a member of the REDACTED's AWERB committee and involved in the organisation of its annual 3Rs event. I am also a member of the Laboratory Animal Veterinary Association and regularly attend their annual conference.

Explain the choice of species and the related life stages

Mice are the species with the lowest neurophysiological sensitivity suitable for these studies. They have been used extensively in immunological research including vaccine development. All of the necessary reagents to undertake the outlined assays are available for mice.





30. Cyclooxygenase in Cardiovascular Disease 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
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

cardiovascular, thrombosis, blood pressure, blood vessels, pharmacology

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo
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 is the aim of this project?

The aim of this project is to understand how cyclooxygenase enzymes work in the body to protect the heart and blood vessels and in inflammation. We want to do this so we can improve the safety and effectiveness of medicines like ibuprofen and aspirin which work by blocking cyclooxygenase enzymes.

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

Why is it important to undertake this work?

Cyclooxygenase is an important enzyme pathway in all organs within the body including those that make up the cardiovascular system. However, we still do not fully understand



how cyclooxygenase works. Medicines that work by blocking cyclooxygenase enzymes are some of the most commonly used in the world and include household names like ibuprofen and aspirin. Aspirin is used to prevent heart attacks and strokes, but does not work for everybody and heart disease remains the leading cause of death in the UK and the rest of the world. Ibuprofen belongs to a family of drugs called NSAIDs which are used to treat arthritis and pain but they can cause heart attacks and strokes in people who need to use them every day. By learning more about the cyclooxygenase enzymes which both kinds of drug work on we think we can make new safer ways to treat arthritis and pain and make aspirin more effective at stopping heart attacks and strokes. In addition, by studying cyclooxygenase we will learn new things about how inflammation and cardiovascular disease develop and new ways to treat these conditions.

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

The ultimate aim of this project is learn about cardiovascular and inflammatory diseases and how medicines that work on cyclooxygenase enzymes (like ibuprofen and aspirin) work. We hope to find new ways to treat these diseases, and ways to use these medicines more effectively and safely. To start, this will be in the form of new scientific knowledge which will be published at the earliest opportunity in scientific journals and presented at scientific meetings. For example, under our current project licence REDACTED. We will always keep in mind how to use this to help patients as quickly as possible. By the end of the project we hope to be able to understand enough to begin to develop some of the following:

Blood tests that can be used to predict which people who take ibuprofen-like drugs will have dangerous side effects. New versions of ibuprofen-like drugs that are safer, especially to the heart and blood vessels.

Better ways to use aspirin and new aspirin-like drugs to prevent heart attacks and strokes.

Completely new types of medicines that could protect the heart or prevent inflammation.

The knowledge gained and publications generated in this project will only be the beginning but will be an important first step in each of these areas. Along the way we also hope to learn things we weren't expecting to find and that may include new ways of performing experiments to refine, reduce and replace the use of animals for medical research experiments.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short term (0-5 years), we will generate new knowledge that will help drive scientific research. We will test our ideas and find new targets that can be the basis of medical tests and treatments to help patients. In the medium term (5-10 years), we can follow this work with studies on humans and patients with disease. In the longer term (10+ years), if these are successful we will be able to turn our basic scientific research into real benefit for patients living with arthritis and cardiovascular disease. Almost 20 million people in the UK suffer from one of these conditions so any new medicines or ways to improve existing ones will have an important impact.

How will you maximise the outputs of your work?



Maximising the outputs of any scientific research depends on communication and collaboration with other scientists and medical staff and keeping the public informed of any progress.

Scientific communication: We will communicate our findings to other scientists as soon as we can and in the highest profile ways possible so our colleagues will be able to contribute to the research. This will include publications scientific journals and presentations at meetings of scientists who work in the area. Importantly, we will make sure all our findings, either positive or negative are shared so that even if we are wrong about some things, other people can learn from this.

Collaboration: We will closely collaborate with other scientists and medical staff to develop the work in the fastest most effective way possible. At the earliest opportunities we will begin to work with medical staff to develop our scientific results into new ways to help patients suffering from disease.

Public engagement: We will also make all efforts to keep the public informed. We will send press releases about things we discover and directly interact with members of the public through events organised at our universities that are open for everyone.

Species and numbers of animals expected to be used

- Mice: 8000
- Rats: 500

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Most animals in our project will be genetically modified but not have any other procedures performed - they will only have samples of their organs and tissues taken for study after they have been humanely killed. Some animals will receive changes to their normal food, be treated with medication added to their water or by injection or very occasionally, have small blood samples taken while they are still alive. At the end of the protocols, animals will be killed humanely straight away or deeply anaesthetised for short surgical procedures to study their heart and blood vessels from which they will not be allowed to wake up.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We have worked hard to design the best possible experiments that will have the minimum effects on animal welfare. The majority of animals used in our project would experience a maximum severity of 'Mild'. This means we would not expect animals to experience more than minor and brief discomfort, for example, a short pain associated with an injection. Our project includes one protocol with 'Moderate' severity where animals would be treated with drugs that cause inflammation and this means they would experience strong flu-like



symptoms where they feel generally unwell. We would not allow them to suffer in this way for more than 8 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 species)?

Mild or non-recovery 90%

Moderate 10%

Severe 0%

What will happen to the animals at the end of the study?

- Killed

Replacement

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

We need to use animals in our project because cardiovascular disease and inflammation are complex processes that involve many different tissues and cells working together. We don't yet understand all these processes, which cells and parts of the body are involved and how they cooperate so unless we study animals as a whole we cannot learn how to prevent and treat these conditions.

What was your strategy for searching for non-animal alternatives?

In our work we use many different types of experiments to answer our scientific questions. This includes experiments on cells grown in the laboratory, experiments on blood and tissue samples taken from healthy people and people with disease and some experiments that are performed virtually using computer simulation or computer analysis of online data. Wherever we can we will use these approaches to replace experiments on animals.

Why were they not suitable?

Unfortunately, we cannot always use simpler approaches in the laboratory with cells or tissues because until we understand the basic processes of diseases we don't know which cells and tissues to study and how to model them. Once we understand these things better we can and will move to replace whole animal experiments with other experiments that don't involve animals.

Another reason we can't always use simple non-animal alternatives in the laboratory is that they cannot show us how all the tissues in the body cooperate to control disease. As an example, we know that cardiovascular disease involves the heart, blood vessels, the kidney and cells in the blood. We can study these separately in the laboratory but unless we study whole animals we cannot see how they work together.

Reduction



Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 we will use in this project by performing statistical power calculations to work out the numbers needed for each experiment and thinking carefully about the total number of experiments we will need to perform. These estimates of animal numbers have also been reviewed by other scientists who work in the area to give us confidence in them.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We carefully plan all our experiments to minimise the number of animals we need to use and get the most scientific information possible. This includes looking at experiments we have done in the past to see how these can be improved and animal numbers reduced, using tools like the NC3Rs

Experimental Design Assistant to plan and organise all parts of the experiment, and taking advice from biostatisticians and other experts in our institute, for example, our REDACTED Statistical Advice Service.

What other measures apart from good experimental design will you use to minimise numbers?

We will take all reasonable steps to reduce the number of animals used in our project. Most of the animals used will be from genetically modified colonies which we will breed carefully at expert facilities in ways that minimise waste and ensure that every single animal can be used in experiments. We will start all our experiments with a small pilot of a few animals to be sure that there will be no unexpected welfare harms and to see if it is needed to go on to a large experiment with more animals. We will collect as much information as possible from every animal for example, making many measurements from the same animal over time. We will also collect tissues from all our animals, and share with other researchers, to perform experiments in the laboratory so no additional animals are required.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?



We will use several different models to induce cardiovascular disease and inflammation and study how these effect the function of the heart and blood vessels. We will use simple models of coronary artery disease and high blood pressure where mice are given added fat or salt in their diet. These are very mild models so don't cause any distress or lasting harm and animals will be killed before they experience any adverse effects of the disease. We will use models of inflammation where mice are treated with substances that mimic a bacterial or viral infection, which can cause symptoms of an infection like flu. We use this because compared to other models it is very easy to control so animals experience the minimum suffering for the shortest time. At the end of the both protocols, we will use surgical procedures that let us directly and accurately measure how the heart and blood vessels function but this will only be done under deep anaesthesia from which animals will never wake up to so they don't experience any suffering or distress.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We will use mice and rats, in the simplest and most humane models possible. In most experiments, animals will be humanely killed without any harmful procedures or procedures will be carried out under deep anaesthesia from which animals will not wake up. We will mostly use mice because they are the least sentient species in which it is possible to manipulate how genes work, to model the diseases we want to study and measure the function of the heart and blood vessels in ways we need to. In a few experiments we will use rats because some blood vessels we want to study are too small to work with in mice.

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We have carefully chosen the most refined models that can deliver as much scientific value as possible with minimum amount of suffering. We will make all efforts to make animals comfortable whether or not they are undergoing an experimental procedure or not, for example, giving them a rich environment to live in and handling them in ways that don't cause them stress. During any experiment, we will closely watch all animals for any sign of discomfort and distress and either stop the experiment or humanely kill any animals suffering unexpectedly. Wherever some suffering is expected from an experimental procedures we will make all efforts to reduce this for example, in the case of animals with inflammation, by keeping them warm to reduce their symptoms or in protocols where animals will be handled a lot, we will get them used to people beforehand to reduce their stress levels. Through the project we will continue to look for new ways to refine our experimental protocols and make animals more comfortable.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will always aim to follow the best possible practice in performing any kind of experiment including those on animals. Although there is no specific guidance published covering the models we use, we will always search and review published information to find the best ways to perform our experiments. More generally, we will follow published best practice guidance for how to design, perform and report experiments to the highest standard including the NC3Rs PREPARE and ARRIVE guidelines and the Centre for Open Science Transparency, Openness and Reproducibility (TOP) guidelines.



How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will constantly review our protocols and experimental design to reduce, refine and replace the use of animals. We will keep informed about any advances by reading scientific publications and speaking with colleagues within our own universities and elsewhere. This will include not only other scientists but those directly involved in the care and welfare of animals. In our REDACTED there are also frequent meetings and training events to discuss the latest developments in the 3Rs and how to implement them including the annual Imperial College Animal Research Forum.

Explain the choice of species and the related life stages

We will use adult mice and occasionally rats for our project because they can be genetically modified and are lowest and simplest species in which we know how to study the parts of the cardiovascular system we are trying to understand.





31. Cytokine signalling 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

Key words

zebrafish, marfan-related syndromes, cancer, therapy

Animal types	Life stages
Zebra fish	adult, juvenile, neonate, embryo, aged
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?

To understand how cytokine signalling orchestrates early vertebrate development and how its deregulation results in human diseases, for example, cancer, fibrosis and Marfan syndromes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 cytokine signalling regulates early vertebrate development will aid in understanding the origin of human congenital abnormalities, and could help develop treatments. Cancer is a major cause of mortality in humans, accounting for about a quarter of all deaths and understanding how deregulated signalling results in cancer could directly result in new targeted treatments. Similarly, for other diseases such as fibrosis (idiopathic pulmonary fibrosis has a prevalence of 50/100,000) and Marfan syndromes (prevalence of 1 in 5000).



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

The outputs of this work include:

An understanding of the role of Activin in cancer and validation of anti-Activin therapy for different cancers, both as a monotherapy and in combination with other therapies. If successful, this could be developed into a cancer therapeutic for humans in the future.

A better understanding of the role of SMAD1/5 signalling in cancer, which could lead to new therapies, and to the development of diagnostic and prognostic markers. Understanding how activating ACVR1 mutations result in DIPG could help with the development of treatments for this fatal disease.

An understanding of how mutations in Ski lead to disrupted TGF- β signalling in Shprintzen-Goldberg syndrome, which could lead to novel therapies. We anticipate that the zebrafish disease model we generate could be used to test novel therapeutics.

An understanding of how mutations in SMAD2 and 3 lead to disrupted TGF- β signalling in LoeysDietz syndrome, which could lead to novel therapies. The zebrafish disease model we generate could be used to test novel therapeutics. Understanding how TGF- β family signalling dictates cell fate specification will be of great benefit to the basic research community and will inform tissue regeneration studies.

Transgenic lines of mice and zebrafish expressing ligand-dependent reporters or expressing biosensors for activated receptors, signalling pathways or transcription factors will be of enormous benefit to the research community as they can be used in numerous other projects where a visual readout of signalling is required.

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

There are likely to be multiple beneficiaries from the outputs above. These include: The anti-Activin therapy we are developing, if successful, will be further developed in collaboration with a pharmaceutical company. We expect to know this within a year. Further development by a company would take of the order of 5 years, before first in human studies. The ultimate beneficiaries will be cancer patients, if we succeed in developing it as a drug.

From our other disease orientated projects, we anticipate that we might discover potential diagnostic or prognostic markers that could be developed for improved clinical management of patients. The time frame for this could be 3–5 years. From our disease models, we may also identify possible treatments that rescue the effects of the mutations, that might ultimately be able to be developed into therapies. The time frame for this could be 5–10 years.

This work will benefit the basic research community by increasing our knowledge of embryonic development and cell biology.

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

A major mechanism to maximise the output of the work, alongside the publication of primary research papers, will be presentation of the work at both big international meetings



and smaller workshops. Not only will the positive results be communicated, but where it is useful for other researchers, unsuccessful approaches will also be highlighted.

We collaborate widely, both internally and externally and this provides another avenue to share details of approaches that were ultimately sub-optimal and how methods were improved.

Where we develop new transformative methods, we will publish specific protocols papers and post on bioRxiv.

Species and numbers of animals expected to be used

- Zebra fish (*Danio rerio*): 77,000
- Mice: 13,120

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.

This project aims to understand how cell communication through cytokine signalling is involved in orchestrating early embryonic development and how its deregulation leads to human diseases such as cancer and Marfan-related syndromes.

For the embryonic development work we use zebrafish embryos that are less than 5 days old. These embryos develop ex utero and are transparent and thus excellent for imaging and we can generate 100 embryos from one set of parents that are synchronous.

For the cancer work, we want to understand how tumours develop and spread and to test candidate therapeutics to inhibit these processes. To do this, it is necessary to work with animals that have similar organs to humans – for example, lungs, pancreas and mammary glands. This leads to the choice of mice. The main cancer types that we study occur in adults, and occasionally, young adults. Therefore, we work with juvenile and adult mice.

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

For the mouse experiments, procedures will be performed that lead to the development of cancer in mice. In the majority of cases, this will be through injected cancer cells. In some cases, mice will develop tumours as a result of a particular breeding protocol. We either simply monitor tumour development and spread when we are investigating the effect of loss (or gain) of a particular protein/proteins, e.g. a component of TGF- β family signalling pathways. Alternatively, mice may receive therapies similar to those being used or being developed to treat patients. Where possible, advanced imaging methods will be used to monitor the spread of tumours and how they react to therapies. Extensive post-mortem tissue analysis will be performed to maximise the information obtained from each animal.

For the zebrafish work, we are predominantly generating either reporter lines expressing biosensors for different signalling pathways, or lines in which we have knocked out or mutated specific proteins in the TGF- β family pathways. These will mainly be studied at the level of embryos and larvae less than 5 days old. Thus the only procedures that the adults go through are breeding and genotyping. In some cases where we are modelling



human diseases like cancer or Marfan-related syndromes, we may study the larvae up to juvenile and adult stages.

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

For the majority of the zebrafish experiments, we do not expect any adverse effects as we are mainly working with transgenics that have no negative effect on development or we are monitoring mutants only in larvae younger than 5 days old. For a small proportion of mutants, there may be some developmental abnormalities. If we observe abnormal behaviour, such as reduced swimming activity or retreat to dark tank corners, the fish will be killed.

In our project mice will develop tumours. Initially, these have little effect on the mice, but as they become larger they might affect mobility. Further, as tumours begin to spread they can affect weight, breathing, and behaviour. Depending on how much the animal is affected, the duration of the adverse effect may range from a small number of days to a small number of weeks.

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

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

For the zebrafish experiments, moderate severity is expected for about 10% of fish. For the mouse experiments, moderate severity is expected for about a third of the 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 have been working on TGF- β superfamily signalling for 20 years and have gained tremendous insights into the mechanisms of signalling and the role of these signalling pathways in regulating cell behaviour from working in model tissue culture systems. However, in order to be able to investigate how these pathways regulate embryonic development, tumour formation and metastasis, and how mutations in pathway components lead to Marfan-related syndromes, we need to perform experiments in animals.

Our developmental work is performed on zebrafish embryos. We have chosen this system because of the rapid embryonic development ex utero and optical transparency allow a direct monitoring and visualization of development, morphology and physiology in the living organism. Recent development of novel genome editing tools (such as the CRISPR/Cas9) system has made it possible to generate mutant zebrafish lines in a highly efficient manner and the technology is easy to implement. Other advantages include ease



of transient genetic manipulation using morpholino oligonucleotides for gene knockdown and sophisticated transgenesis tools (such as the Tol2 transposon system) to generate fluorescent reporter lines. For our zebrafish work, to minimise the number of procedures performed on animals deemed to be sentient, we do most of our work with zebrafish embryos and young larvae, before the onset of independent feeding. The only work that will be done on adult or juvenile fish is the fin clipping for genotyping, transient warming to induce expression of transgenes, and gamete harvesting. Concerning the genotyping, as often as possible mating will be performed followed by analysis of phenotype to determine the genotype of the fish as an alternative to fin clipping.

With regards to the mouse work, we only perform studies on mice that cannot be done in model tissue culture systems or in the zebrafish system. Moreover, we also study tumourigenicity *in vitro*. If our data show a good correlation between *in vivo* tumourigenicity and the *in vitro* properties of given cell lines and organoids, we may be able to further limit the number of tumourigenicity experiments.

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

We complement our *in vivo* analyses with tissue culture models and organoids.

Why were they not suitable?

We can gain a certain amount of information from these *in vitro* systems, but there are serious limitations. It is not currently possible to replicate the complexity of mammalian tissue structures in culture models. Moreover, the immune system only functions effectively in an organismal context with appropriate white blood cell movement and function within lymph nodes. Finally, in the process of metastasis the cancer cells move between organs. It is not possible to recreate this using *in vitro* models. One possibility to study tumour cell invasion is to perform some of these assays in young zebrafish larvae, before the onset of independent feeding. It is possible to gain information about the ability of particular tumour cells to exit the circulation and invade tissues. This may reduce the number of mice required in the project.

Reduction

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

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

For the zebrafish work we calculate the numbers of fish required based on the numbers of mutants and transgenics that we are currently maintaining, plus those that we need to generate to be able to achieve the aims of the project. Details of how many fish we need to raise to generate mutants and transgenics are given below. We currently maintain 30 mutant lines and 15 transgenic lines. We estimate that in the next 5 years we will generate around 20 new mutants and 7–10 new transgenics.

For the mouse work, the estimate is based on several factors. Firstly, we are using our previous experience of the last 5 years and the experience of our collaborators who use similar approaches with the same mouse lines. Secondly, we have taken into account the



number of researchers within the group who perform mouse experiments (currently 3, and likely to be between 3 and 4 in the next five years). We also continually re-evaluate the numbers of mice required for each experiment using power calculations. For this we access help from an in house statistician when necessary. This will allow us to determine the number of animals required per experiment. Numbers of mice used for breeding are based on best practice and we maintain between 10-12 strains.

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

For the zebrafish work we regularly review our mutant and other stocks and cull any that are no longer required. For all zebrafish lines, we freeze sperm to archive the line. Thus, we will only maintain lines in the aquarium that we are actually using in on-going experiments. Through exchanges with other zebrafish labs, in the UK and elsewhere, we will be able to minimise the number of mutant and genetically modified strains that we keep in our own aquarium. Moreover, the stocks of adult mutant fish that we will keep will almost all be heterozygotes carrying recessive mutations and thus phenotypically normal. In addition, because we share the fish aquarium with a number of other labs, we ensure that multiple workers perform their experiments on the same day to maximise the use of the embryos from a given batch of fish. Any extras are fixed for subsequent experiments. We get on average 100 embryos per pair of fish.

For the mouse work experiments are designed to not falsely detect effects and not miss effects. Based on our previous experience of how variable our measurements are and how big the effect we are looking at is, then most experiments involve around 5-10 mice per group and 3-7 groups. If the necessary in vivo data does not exist, then experimental design will be informed by a combination of prior in vitro data generated in the laboratory, existing publications, and previous experience.

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

For the maintenance of zebrafish lines, we estimate that we use 400 fish per line over 5 years.

For generation of new transgenic lines, estimates of numbers of fish needed are based on statistics on the efficiency of transgenesis from ours and other laboratories. Typically for one transgenic line we will raise 100 F0s, which will be screened by crossing them to wild type and screening their progeny for the transgene. Typically 20-30 of the adult F0s will be founders. We will choose 2-3 of these and raise their progeny; the others will be culled. For each 'family' we will be raising between 50 and 80 fish which will be genotyped to identify heterozygous carriers of the transgene. The transgenic lines will be then maintained as for other wild type and mutant lines, which corresponds to about 4 tanks of around 20 fish each.

A similar strategy will be employed for the mutant fish generated by TALENs or CRISPR-Cas9 technology. To produce one mutant line we raise 200-250 ('founder fish'), which are phenotypically normal after microinjection of the CRISPR/Cas9 constructs or TALENs. To screen for germline transmission and loss-of-function alleles the F0s will be crossed to wild type fish, and their progeny will be screened as embryos for the presence of the mutation. We will pick 2-3 positive founders and raise 200-250 progeny. The others will be culled. The F1s will be screened for the heterozygous mutation by fin clipping. Heterozygous F1s will be further outcrossed to wild type fish to segregate away any nonspecific mutations



and in-crossed to other transgenic lines (mutant lines to generate double mutants, GFP-transgenic reporter lines). We will raise 100-150 F2s from these crosses and screen them for the desired heterozygous mutations. The mutant line will be then maintained as for other wild type and transgenic lines, which corresponds to about 2-4 tanks of around 20 fish each.

For the mouse work we will try to keep as few mice as possible by careful monitoring our mouse colony and good practice. To minimise breeding, lines under sporadic use are maintained at lower levels, and frozen whenever practicable. Lines will be maintained in collaboration with other licensees wherever possible to minimise redundant breeding.

Our mouse lines are routinely maintained by keeping 2-3 breeding pairs, with around 3-4 litters/year - total 75-100 animals per strain/year. For crosses to enable characterisation of specific phenotypes, in general 5-6 breeding pairs will be kept with 6-8 litters/year - total 350-400 animals per strain/year. For mouse cancer models involving multiple alleles, such as the KPC mouse model of pancreatic cancer, where only approximately 1 in 4 mice will get tumours from an in-cross, then 10 breeding pairs will be kept with 6-8 litters/year – 700 mice per strain per year.

In our mouse experiments designed to test the effect of therapeutics on cancer development and spread, we are moving towards the use of transplantation models into syngeneic mice, rather than relying on spontaneous tumour models. Particularly in the case of the pancreatic cancer models, this reduces the numbers of mice that need to be bred for the experiment.

Refinement

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

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

We use the zebrafish model for the vertebrate developmental work, as these embryos develop ex utero and can be manipulated genetically and are transparent, and thus ideal for imaging.

All embryological work is preceded by in vitro or tissue culture studies to test the approach or activity of the biological entity to be introduced into the embryo. Failure at this preliminary stage is taken as final and no in vivo work will take place until this step is successful. In addition, for the zebrafish we will minimise suffering by taking pains to maintain the general health of the fish population, by attention to water quality, feeding regimes, and fish population density in each tank. We will check all breeding stock daily and cull any that show signs of significant illness or deformity. Where surgical or other potentially distressing procedures are required, e.g. fin clipping, we will perform them under general anaesthesia with analgesia both pre and post fin clip. Any fish or fish larvae showing signs of distress on recovery from a surgical or other procedure will be killed promptly by an approved method.



We will use mice in this project to investigate tumourigenesis in a mammalian system and to test new therapies. For the generation of breast tumours, we will most often employ sub-cutaneous injections or injections into the mammary fat pad. Using syngeneic transplant we have better control of tumour growth dynamics, than using a spontaneous genetic tumour model, where tumours arise at multiple sites sporadically. This reduces mouse numbers as 1 tumour from a genetic model can be used to generate tumours in around 15 mice. We use ultrasound-guided injection into the pancreas to study pancreatic cancer. Similarly, this has advantages over a spontaneous genetic tumour model, as we have more control over the time of tumour development. For the study of lung metastases, intravenous injections will be the route of choice. On rare occasion, we will study metastasis to other organs and therefore use other injection routes, such as intra-cardiac to give bone metastases. For skin tumours, we will use a topical carcinogen application. We are also using genetic models for breast cancer to mostly to generate tumours for transplantation assays. For the pancreatic cancer model, we are using spontaneous genetic tumour models. The parental lines are maintained at a minimal number and only bred in larger numbers for particular experiments.

For the mouse work we will check the animals every day and kill any exhibiting signs of significant illness. We will also reduce the overall tumour burden on mice and thus minimise possible adverse effects, by for example using the non-invasive imaging for metastasis assays.

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

We use zebrafish embryos for our developmental work, and most of this is on fry less than 5 days old, which are deemed non sentient and before the onset of independent feeding. For the tumour work, we will try to use zebrafish larvae to investigate tumour cell invasion and extravasation. This will inform our mouse experiments and should reduce the number of mice needed. We also use terminal anaesthesia to obtain very detailed information about tumours. However, to understand the efficacy of therapy it is necessary to analyse the same tumour before and after giving treatment. Where possible we will use on non-invasive longitudinal imaging.

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

We try to minimise any possible adverse effects. In particular, the use of fluorescently labelled cells coupled with microscopic analysis of tissues enables us to detect small metastases. This reduces the overall tumour burden needed to be able to detect metastasis from primary sites. Similar benefits are expected from the use of non-invasive fluorescent or bio-luminescent imaging. If the purpose of the experiment is simply to observe cellular behaviours in the primary tumour, then we would not grow the tumours to the larger size that some of the metastasis experiments require. Regular monitoring by BRF staff is in place and we strive to keep updated with the latest environment improvements, such as enhanced environmental stimulation.

Surgical procedures will be performed with suitable anaesthesia and animals monitored post-surgery to ensure that they recover well. We will also use suitable analgesia for all surgery.

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



We are aware of NC3Rs. We also discuss with colleagues in other research groups new improvements that lead to refinement.

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

We will stay up to date via regularly communication with BRF staff, other scientists in the field and regular visits to the following website <https://www.nc3rs.org.uk/3rs-resources>.



32. Redacted

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

The aim of this study is to examine the host immune response to infection REDACTED

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

Why is it important to undertake this work?

REDACTED is a severe disease of sheep and has a significant impact in areas where it is endemic, both animal and human welfare. Most of the areas where this disease is reported to be endemic are in REDACTED. The disease can cause high levels of morbidity and mortality in affected animals. There is no vaccine, which is the ultimate goal for disease control. Sheep which are present and survive in endemic areas appear to be immune to the disease, however very little research undertaken to understand the host immune response to infection (and vaccination) with REDACTED. This work will examine and define the mechanisms of immunity of the host to REDACTED, the information from which will be used in future to develop vaccines and other countermeasures to REDACTED.



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

Defining the sheep immune response to REDACTED infection, with an eventual development of a vaccine, would be a major step forwards in controlling this disease in sub-saharan Africa. As well as being a disease which can kill up to 90% of sheep, the damage such losses can cause to the human communities which rely on these animals for food and economic prosperity can be catastrophic. Development of countermeasures to such a disease would increase productivity in endemic areas, and reduce the consequences of widespread loss of animals.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Publication of the data generated upon completion of this study will allow fellow researchers to further develop vaccines and countermeasures to REDACTED. Long term application of the fundamental host immune responses to infection with REDACTED could be used to screen vaccine candidates in the host without the need for challenge, both refining and expediting REDACTED vaccine development in sheep. The data generated, and further definition of the sheep model to REDACTED infection can also be used to further investigate vaccine development to CCHF infection of humans (a lethal haemorrhagic disease virus).

How will you maximise the outputs of your work?

The data generated from this study, whether successful or unsuccessful will be published in peer reviewed journals to enable the immune responses to REDACTED infection to be disseminated.

Species and numbers of animals expected to be used

- Sheep: 51

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

To define the immune responses in the sheep, the animals will need to be vaccinated (experimental vaccine) and / or infected with REDACTED.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The vaccination of the sheep with the vaccine is not expected to cause any adverse effects other than short lived discomfort / elevated temperature as is normal with any vaccination, and is considered to be of a mild level of severity. The infection of sheep with



the NSD virus is likely to cause an elevated temperature, discharge from the nose and eyes, and diarrhoea which may have traces of blood.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

These signs will be of a Moderate Severity. The Animals will be culled at the end of the study, or if they become too sick during the study, using the most humane methods of culling.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Sheep are the target species for this virus, in that they will be the species in which the information generated will be used to assess immunity to vaccination / vaccine development in future. Due to the complex nature of the immune system, it is not currently possible to ascertain what component of the immune system is responsible for clearing an infection and protecting against future infections.

What was your strategy for searching for non-animal alternatives?

Cell culture based systems to amplify the virus will be used in place of using animals to amplify the virus. Dose rates will be derived from previously published studies. Whole animals need to be used to assess the immune response to the virus and / or vaccine

Why were they not suitable?

No replacements options are available to replace the whole animal at this time as an entire organism, including the immune system, need to be present to assess the immune response of an animal to a virus 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?

We will use statistical tools to determine the minimum number of animals that we will need in order to achieve our scientific aims and objectives, outlined above, with a high level of



certainty. This number of animals has been selected assuming 4-5 animals per group, with 3 dose rates to define the challenge dose required to illicit an immune response. This also includes 5-6 animals per vaccine group with 3 doses of vaccine. This also includes 3 groups of 6 animals (naive, exposed to low dose of virus, and one vaccinated group) for subsequent challenge with virulent dose of virus. This equates to 51 animals.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The site statistician has been consulted and assuming similar response rates as other animals infected with similar viruses / vaccinated with similar vaccines, the estimated numbers would be the minimum to achieve the scientific aims.

What other measures apart from good experimental design will you use to minimise numbers?

Given very little is known about the immune responses of sheep to REDACTED, we will collect and store tissues to be analysed and shared by other scientific groups from both samples required in this study, but also after asking other research groups.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

As explained, sheep as the target species, must be used as the model species so we have confidence that the data generated will be applicable to other sheep. There is no other animal model for REDACTED infection. The experimental vaccine used in this project to help further define the immune response to REDACTED will have first been screened in mice to ensure it has the potential to generate an immune response before it is trialled in sheep. We will only challenge animals with virulent REDACTED to assess protection once we have analysed the immune response to both vaccination and prior infection and are confident that there is a good chance these animals will be protected.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

There are no other animal models for REDACTED infection, although mice will be used to screen the vaccine for immunogenicity before it is used in sheep.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals will be housed together with bedding and other items of enrichment. Highly trained animal technicians will monitor these animals throughout the day, ensuring they



are comfortable and to maximise their welfare status. We have 24/7 CCTV surveillance which can be used to monitor the animals behaviour over time.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Adherence to the ARRIVE guidelines for reporting these studies, as well as to the FELASA guidelines for large animal health monitoring to help ensure the most robust health assurance for animals used in this study.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Through continued CPD and frequent review of the CAAT (Center for Alternatives to Animal Testing) I will keep informed about advances in the 3Rs. Included in CPD will be annual attendance at national lab animal science conferences as well as naturally reviewing the current literature surrounding infectious disease research, in particular Nairoviruses.

Explain the choice of species and the related life stages

These animals, sheep, are the natural host for REDACTED and no other animal models exist. Adult sheep are used as this best reflects the life stage of sheep which are naturally infected with REDACTED.





33. Defining the link between parental nutrition and offspring development

Project duration

5 years 0 months

Project purpose

Basic research

Key words

Parental diet, Gamete quality, Developmental programming, Mouse model

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The aim of this project is to understand the impact of parental diet on reproductive fitness in mice. Additionally, this project aims to define the long-term effects of poor parental diet on offspring 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?

Developing a clearer mechanistic understanding of the link between paternal diet and offspring development is of fundamental importance for preventing life-long offspring ill-health. The research conducted under this project will provide new insight into the importance of parental diet at the time of conception for the health and well-being of offspring. This project will inform how poor diet impacts on the metabolic and reproductive health of the parents, and both the short- the long-term health consequences for the offspring. This study will define new biomarkers of sperm and oocyte quality, post-fertilisation development and offspring growth in relation to parental diet. Such data will be of value for human and commercially important animal species, as well as helping to



inform the development of future nutritional guidelines for prospective parents to support directly healthy ageing across the life- course.

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

With the increased global prevalence of heart disease, obesity and diabetes, a greater understanding of how adult ill-health is determined has never been of greater importance. This novel study will provide new insight into the significance the diet of the parents have on the development and long-term health of their offspring. Under this project, the underlying biological mechanisms regulating heart disease, obesity and diabetes in adult offspring of dietary manipulated parents will be defined. Such insight will provide scope for the development of new interventions and treatments to alleviate their effects and benefit human health. In addition, this study will identify the interaction between sperm and eggs, both from parents fed a poor diet, to determine their combined effect on offspring development.

It is anticipated that several publications (both primary research and review articles) will be produced by the end of this project. Data generated under this project will have been presented at multiple national and international conferences by the principal investigator, post-doctoral researchers, PhD and graduate students.

Furthermore, this project will result in the generation of a significant bank of tissues which will act as a valuable resource for subsequent graduate project students, PhD students and collaborators. This tissue bank will also aid in the generation of preliminary data for subsequent grant applications. Furthermore, through interdisciplinary sequencing, lipidomic and proteomic analyses of these tissues, non-animal based bioinformatic and mathematical modelling research approaches can be developed reducing further need for animal research.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The results generated from this proposal will have broad benefits to many diverse groups within the academic community. This study will define the impact of parental diet on sperm/egg epigenetic status, embryonic development, fetal growth and adult offspring health. As such, this study will re-define our understanding of the early origins of adult ill-health, providing novel insight into the significance of poor parental diet for the development and wellbeing of offspring.

The findings from this project will be of significant benefit and interest to those involved in assisted reproductive technology (e.g. IVF) research. This study will provide essential insight into the consequences of using sperm and eggs from individuals on sub-optimal diets, providing novel biomarkers of perturbed post fertilisation development and their impact on subsequent fetal growth. Data from this proposal will help define new assisted reproductive technique methodologies and strategies for gamete handling, manipulation and usage, as well as preconception advice for couples entering the clinic.

Wider research communities will benefit from the resources generated within this project such as the development of novel mechanisms for analysing embryonic gene transcription, deposition of novel sequencing data sets within online repositories (e.g. NCBI Gene Expression Omnibus), the development of new software pipelines for analysis of gene



expression profiles with associated epigenetic modifications and the impact of parental diet dietary modifications on epigenetic inheritance and offspring development.

Finally, findings from this proposal will be of benefit to health-care professionals offering dietary advice to intending parents, clinicians involved in assisted reproduction and fertility treatment, paediatricians, policy makers concerned with diet, adult health, fertility and pregnancy management.

How will you maximise the outputs of your work?

To maximise the output from this project, we will publish the research data in high-impact, open access journals. Furthermore, data collected under this project will be presented at international, national and institutional conferences, meetings and seminars. In addition, we will use publicly accessible outlets such as the Conversation UK to discuss research originating from this project.

Where ever possible, we will also collaborate and share resources (samples, knowledge, technical skills) with other researches (both within and outside of the REDACTED) to maximise the scope and impact of our research.

It is our anticipation that all data generated under this project (both positive and negative) will be made available.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Under this project, we will define how parental diet affects their reproductive fitness, the development of the early embryo, fetal growth and offspring metabolic health. As such, two groups of animals will be studied under this project, parents and offspring. In both cases, both males and females will be studied.

Typically, parental mice (males and females) will be fed *ad libitum* either a control or an experimental diet (either low in protein or high in fat). For males, these diets will be given for several weeks prior to mating, while females will be fed for a specific duration around and during pregnancy (typically between 3 days and 3 weeks in total). Typically, these mice will then either be (i) culled for the isolation and analysis of their sperm/eggs (ii) mated to analyse how their diet affected the development of (a) early preimplantation embryos, (b) fetal growth or (c) the postnatal health of their offspring or (iii) receive embryos that have been generated *in vitro* and which will be allowed to develop to different stages of pregnancy or term. Females receiving donor embryos will typically be mated to vasectomised males (to render them pseudopregnant) prior to embryo transfer (either surgically or non-surgically).



While males and females are being maintained on their diets, it will be of interest to assess how their diets are affecting their metabolic health. This will typically involve glucose tolerance testing, where mice receive an intraperitoneal bolus of glucose followed by regular collections (over 2 hours) of blood from the tail vein for measurement of blood glucose and/or insulin levels. Additionally, males and females may undergo positron emission tomography scanning for in vivo analysis of metabolic uptake. This will involve injecting mice with non-toxic, non-radioactive stable isotope labelled metabolites (e.g. Fludeoxyglucose; ^{18}F), being anaesthetised and then non-invasively scanned over a 2-3 hour period to measure the uptake of the metabolites into their tissues prior to full recovery.

Analysis of offspring (males and females) growth, reproductive fitness and metabolism will be conducted in a manner similar to their parents i.e assessment of glucose tolerance, metabolic uptake using positron emission tomography scanning and isolation of sperm, eggs and embryos. It may also be of interest to study how offspring from parents fed a low protein or high fat diet respond metabolically when also fed the same poor quality diet. Under these experiments, offspring (both males and females) will be fed either control or experimental diets in a manner similar (duration and stage of gestation) to that of their parents.

At the end of all experiments, all mice (parents and offspring) will be culled for the isolation and storage of tissues for additional analyses such as gene and protein expression, hormone levels and tissue morphology.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Animals fed experimental diets may experience significant changes in their metabolic and reproductive physiology. These may include significant gains in weight, underlying changes in blood glucose and lipid levels and reductions in sperm/egg quality and embryo development. It is anticipated that these physiological changes will last for the duration of the feeding regimen. There is also the possibility that a very small number of animals will find their diets unpalatable. In this instance, mice may experience some weight loss. However, these effects are not expected to result in significant pain, suffering or distress to the animals or their well-being or general condition.

During the project, several groups of mice will also receive intraperitoneal injections of various substances such as glucose, hormones or stable isotope labelled metabolites. These will typically be single injections which may result in inflammation at the site of injection. Similarly, for some animals, we will want to take blood samples from a tail vein for the analysis of metabolite or hormone levels. This may also result in inflammation and/or soreness at the site of sampling. However, the impact will be localised to the site of injection and the duration should typically be no more than 24 hours.

Animals undergoing surgical embryo transfer may experience adverse effects from the anaesthesia (respiratory distress) or from the surgery itself such as post-operative infections, pain and/or wound breakdown. These effects are likely to occur during or shortly after the surgical procedure and will be mitigated for by constant monitoring of the animals and administration of appropriate pain relief. In worst case scenarios the animal may be culled.



Animals placed within the metabolic cages may experience stress due to the social isolation and minimal environmental enrichment. However, the effects are anticipated to be transient and all animals will be returned to their home cage and monitored or culled immediately after the cessation of the experiment.

Finally, some animals may be anaesthetised and injected with non-toxic, stable isotope (on-radioactive) labelled metabolites for the analysis of how these are taken up and used by the body. During anaesthesia, some animals may experience adverse reactions which may involve regurgitation of their stomach contents or difficulties in breathing. If this occurs, these mice will immediately be removed from their anaesthetic and allowed to recover.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

Over the duration of this programme of work, it is anticipated that 90% of animals will experience procedures that are of a mild severity nature. The majority (70%) of these will be placed on diets of modified composition. It is expected that feeding animals the control, low protein or high in fat diet will result in mild severity changes to their weight, metabolism and/or reproductive fitness. However, in our experience, none of our experimental diets result in increased mortality, or the need to cull any mice, even after several weeks of feeding.

It is anticipated that 25% of the animals will receive injection of substances for the analysis of glucose tolerance, hormonal induction of ovulation or for the analysis of metabolic status by positron emission tomography. These will typically result in mild severity short-duration effects on all animals. Effects will typically be in the form of soreness and swelling at the site of injection and are likely to occur in all animals receiving injections and are of a mild severity.

Animals placed with metabolic cages (approximately 10% of all animals) will experience mild severity effects due to the isolation and minimal environmental enrichment. However, the effects of this will be temporary and are not anticipated to result in impairment of the well-being or general condition of the animals.

All animals undergoing surgical embryo transfer (approximately 10% of all animals) may experience moderate severity, short-term effects from the anaesthesia and surgery. However, these will be mitigated through the use of appropriate pre- and post-operative pain relief and constant monitoring.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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



The purpose of the work is to determine the effects of parental nutrition on sperm/egg quality, embryo development, fetal growth and adult offspring health. The manipulation of human fetal and embryonic tissue for the sole purpose of understanding their impacts on long-term offspring development and well-being would be considered un-ethical. Therefore, to achieve the aims of this project, the use of animals is necessary to cover the complex, inter-related, whole body nature of the study.

What was your strategy for searching for non-animal alternatives?

The aim of this study is understand how parental diet affects a multitude of reproductive, developmental and adult offspring phenotypes. As such, whole animal studies are required and can not be replaced in their entirety by *in vitro* alternatives such as cell culture, organoids, 'tissues-on-chips' or *in silico* analyses of published data sets. Furthermore, as non-mammalian species such as insects, worms or fish have dramatically different patterns of reproduction to humans and mice, their use in this project would not be conducive to answering the aims of this project.

However, where possible, individual experiments will be conducted *in vitro* (sperm morphology and motility, pre-implantation embryo development, isolated tissue function analyses) as well as numerous molecular and biochemical assays on collected tissues (gene and protein expression patterns, serum hormone and metabolite levels) thus minimising the need for repetitive experimentation on animals and maximising the data collected from any one individual.

Why were they not suitable?

A central component of this project is to examine the effects of diet on integrated, whole body systems e.g. parental reproduction, fetal growth, adult offspring metabolism. While complex *in vitro* systems have been developed to study central biological processes e.g organ on a chip, organoids, 3D- scaffolds for cell culture, these models represent isolated systems *in vitro*. Therefore, in order to understand how diet affects parental reproduction, growth metabolism, hormone balance (all interconnected) in an undisturbed system, live animal research is necessary.

Reduction

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

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

I have determined the number of animals to be used in this project through the use of the NC3R's online Experimental Design Assistant and my existing body of mouse model data. Using these, I have calculated the necessary number of offspring litters required to observe statistically significant differences in the phenotypic measurements outlined. Animal numbers are provided below to estimate offspring replicates required based on an average litter size of 6 offspring per litter.

For the generation of 50 litters of adult offspring (10 litters per dietary treatment group; 1 control and 4 experimental with approximately 6 offspring per litter) 70 adult stud males



and 80 adult females (allowing for natural redundancy based on existing studies) will be required. From my previous studies, an average litter size of 6 offspring is anticipated. However, this number can vary naturally and some litters, if lost during gestation or at birth, would need replacing. However, the number of litters lost and which require replacing is typically low.

For the generation of sperm, oocytes and embryos for analysis, a further 100 stud males and 150 females will also be required throughout the licence.

Vasectomised males (150 in total) will be purchased from a commercial supplier and used in batches of 50 (five treatment groups comprising 10 separate males in each) throughout the lifetime of the project.

For embryo transfer experiments, both donor and recipient female mice will be required, with a greater number of recipient females needed due to anticipated success rate of 60%. Therefore, it is anticipated that 200 donor females (100 naturally mated and 100 superovulated) and 150 recipient females will be needed.

Similar number of offspring litters are required as for the analysis of fetal development. This will require 10 litters per treatment group (1 control and 4 experimental with approximately 6 offspring per litter). As for the fetal analyses, some natural redundancy will occur and some litters will need to be replaced. Furthermore, in some studies, a small number of F2 generation offspring will be generated. As such, an anticipated total of 500 offspring will be generated under this project.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Statistical advice was sought to devised an analytical approach which allowing us to take into account the multiple levels of interaction within our data and other potential confounding variables (e.g. parental origin, litter size, body weight etc). Using this approach we are able to factor into our statistical analyses the multiple use of any one male in more than a single experiment. Such analyses allow us to use stud males several times, reducing the number of stud males required.

In addition, we have used the NC3Rs experimental Design Assistant to confirm that an n of 10 litters per treatment group are necessary to observe significant changes in fetal growth (a major determinant of long-term ill-health) based on our existing data.

What other measures apart from good experimental design will you use to minimise numbers?

Where possible, stud males will be mated on multiple occasions prior to culling for the collection of tissues for subsequent molecular and biochemical analyses. This will ensure maximal data is obtained from each stud male and will minimise unnecessary repetition of experiments.

For our analysis of metabolite uptake using positron emission tomography we will conduct pilot studies to ensure optimal concentration of stable isotope labelled metabolites to be injected and the duration of the imaging necessary. Such pilot studies will ensure animals are maintained under anaesthesia for the minimal duration and prevent unnecessary repetition of experiments.



From all animals used within this project, a range of tissues will be collected and stored for later analysis. These will be used for under-and post-graduate studies within our laboratory, for the generation of preliminary data for further grants and will also be made available to collaborators where appropriate.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice represent the most appropriate animal model as their use maintains continuity with my existing experiment data and will enable me to define the underlying biological mechanisms in unparalleled detail. Mouse reproductive physiology, mechanisms of sperm/egg maturation, patterns of embryonic and fetal growth and regulatory mechanisms for adult metabolic health share significant similarity with that of humans. Non-mammalian species such as insects or fish are entirely inappropriate since they do not include internal fertilisation and embryo development, stages of fetal development or the presence of a definitive placental. As all these aspects will be investigated under this project, use of a mammalian species is essential. Furthermore, the use of mice, and especially the C56BL/6 strain, permits detailed investigations into the genomic and epigenomic mechanisms linking paternal diet with offspring health. The unparalleled annotation of the C56BL/6 genome, transcriptome, proteome and epigenome will allow for maximal levels of data retrievable from each animal. For these reasons, rodents provide the most appropriate non-human model for such studies.

During surgical procedures, animals will receive appropriate pre- and post-operative pain relief. Surgery will be conducted under sterile conditions using properly calibrated and maintained equipment for the administration of inhaled agents. To prevent unnecessary surgical procedures on female mice, where appropriate, the Non-Surgical Embryo Transfer (NSET) device will be used for both artificial insemination and embryo transfer.

Under this project we will use positron emission tomography (PET) scanning to determine the impact of parental diet on parental and offspring metabolic health. Typically, in order to analyse an individual's metabolic status, mice have to be culled for the isolation and analysis of specific tissues. However, positron emission tomography (PET) scanning, using non-toxic, non-radioactive analogues of key metabolites such as fludeoxyglucose (18F-FDG PET), is an increasingly used technique in murine models of human diseases. Under several physiological and pathological conditions, the use of PET can be used to look at the uptake of labelled metabolites such as glucose, amino acids or lipids into tissues. As real-time *in vivo* measurements of metabolite uptake can be conducted on live, anaesthetised animals, this represents a significant refinement over studies in which animals need to be culled.

In conjunction with the use of PET scanning, we will also use the Columbus Instruments system (CLAMS) metabolic cages. Use of this system will substantially reduce the number



of animals required for experimental procedures and improved the quality of the experimental data as multiple measures are recorded simultaneously from the same animal. However, these metabolic cages provide a sparse environment as compared to the animals' home cages, thus further refinement will occur to a) minimise the time needed to be spent in the metabolic cage to obtain satisfactory data, and b) to determine whether providing environmental enrichment whilst in the metabolic cage is possible (for example baseless housing, provision of gnawing blocks) without disrupting the quality of the metabolic and behavioural data captured.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Patterns of non-mammalian reproduction display dramatic differences to that of humans. Patterns of sperm/egg development, fertilisation, fetal growth and offspring development are significantly different to humans. Furthermore, non-mammalian species do not possess a placenta, a key organ in the regulation of fetal growth. As such, in order to make the findings from this project relevant to human health, a model system that displays close reproductive and developmental similarities with humans is needed. As mouse reproductive physiology, fetal growth and offspring development share significant similarities with humans, they represent an optimal model for these studies.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

All animals placed on experimental diets are monitored weekly for weight gain/loss, general condition, demeanour and behaviour. Any significant deviations from normal will be recorded and, where appropriate, result in discussions with staff within the facility, the NVS or their deputy and appropriate action enacted.

All animals undergoing anaesthesia and surgery will be monitored daily for up to 72 hours post-procedure. This monitoring will assess welfare aspects such as general appearance, appearance of wound (where appropriate), spontaneous behaviour, provoked behaviour and weight. This monitoring will take the form of a 20 point scoring system with animals being rated as either (i) normal, (ii) requiring close monitoring with possible analgesia, (iii) requires humane culling and careful consideration of the protocol. Animals undergoing mild severity protocols with a score of 2 or greater in any category, or a cumulative score of 5 or more on the scoring sheet will be culled humanely. Animals undergoing moderate severity protocols with a score of 3 or greater in any category, or a cumulative score of 8 or more on the scoring sheet will be culled humanely. This monitoring system has been approved for use by our institutional AWERB and is currently in place.

In addition, to minimise the impact of post-operative pain in any animal, mice will be given a suitable pre-emptive analgesic and post-operative reactive analgesia as advised by the NVS or their deputy. To minimise further the welfare costs of the embryo transfer procedure, where possible and experimentally appropriate, we will use non-surgical embryo transfer procedures such as using the NSET device.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Throughout the course of this project we will make reference to multiple sources for best practice guidance. these will include:-



Fund for Replacement of Animals in Medical Experiments: www.frame.org.uk
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>
Laboratory Animal Science Association (LASA) publications:
https://www.lasa.co.uk/current_publications/

We will also keep up-to-date with the latest non-animal research models to ensure, where possible, non-animals based experimental models are adopted

Altweb: <http://altweb.jhsph.edu/>

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I currently subscribe to the NC3Rs monthly e mail alerts which keep me updated to 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.

Finally, information is disseminated and methods of best practice are discussed via our establishment users meetings of which my research group are involved with.

Explain the choice of species and the related life stages

Mice represent the most appropriate animal model for these studies for a number of specific reasons. Firstly, their use maintains continuity with my existing published research and permits us to make direct comparisons between our previous data and current studies.

Second, patterns of mouse oocyte and pre-implantation embryo development are significant similar to humans, making them the most appropriate model of understanding the environmental effects on embryo development. Non-mammalian species are entirely inappropriate since they do not include fetal development.

Third, the un-surpassed genomic annotation of the C56BL/6 strain permits analyses into the effects of parental nutrition upon offspring gene expression and epigenetic regulation, maximising the level of data retrievable from each animal.

Finally, the use of rodents allows for the isolation, culture, manipulation and cellular analysis of all all stages of the reproductive and developmental cycle. As such, by using rodents it is possible to understand the entire time-line looking at gametogenesis, embryo development, fetal growth and adult offspring health. Such studies are not possible in humans or non-mammalian species.

For these reasons, rodents provide the most appropriate non-human model for such studies into the effects of parental diet on sperm/egg quality, fetal growth and offspring health.





34. Determining the uptake and toxicity of plastic particulates in fish

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

No answer provided

Animal types	Life stages
Zebra fish	adult, juvenile
Salmon	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 is the aim of this project?

The aim of this project is to provide essential information surrounding the dietary and waterborne exposures of fish to plastic particles in the size range of 1 nm to 5,000 µm. We aim to better understand the influence of particle characteristics (e.g., size, shape, polymer type and presence of co- contaminants) on the uptake and toxicity in fish.

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

Why is it important to undertake this work?

Plastics are lightweight, durable and inexpensive materials that are used in a wide range of consumer products. Global plastic production is above 335 million metric tons, of which around 40% was single use. Once released into the environment, heat and ultraviolet light can cause larger plastics to fragment into microplastics (<5mm), with concerns about the further degradation producing nanoplastics (<100nm). Environmental sampling of pelagic fish species has shown the presence of microplastics in the gastrointestinal tract. Of the



laboratory studies conducted on fish, much of the work has focused on using embryos and/or short term exposures. Due to this, key questions surrounding the influence of exposure pathway on the uptake and toxicity of plastic particles in adult fish is lacking. Specifically, it is important to gain information surrounding the influence of particle characteristics (e.g., size, shape, polymer type, presence of co-contaminants) to inform risk assessment. The current project is important because the data will be used to inform a geospatial risk map to guide policy and management decisions for plastic pollution.

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

One outcome of this project is an enhanced understanding of plastic particulate uptake and toxicity in fish with regards to:

The role of exposure pathway.

The role of physical particulate characteristics.

The role of chemical co-contaminants vectors.

Each of the three objectives should produce at least one primary publication.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The significance of this project has been described in detail in a previous section (Background). There are 4 main beneficiaries to this work:

Other members of the research team will have access to the data. This will be of particular use to members who are investigating invertebrate systems to compare species sensitivity. This data will be available to other members as it is produced.

Chemical regulators as established methodologies are being followed so data between studies can be compared. This data will be compiled into publications, aiming for open access journals.

The data will inform on geospatial risk map of plastic pollution of shelf seas around the UK. The beneficiaries will be manufactures of plastics because we are going to increase their awareness of the problems their waste creates. This might inform on what they will do to minimize it. As we aim to publish data that is open access, this information should be of use to environmental regulators in guiding policy. The experimental data to be used in this risk map will be collected in the life of the project.

Industrial stakeholders who use plastic particulates, or larger plastics which can breakdown through the product life cycle or environmental degradation. The benefit here could be to convince plastic users to choose a more sustainable ways of use or material.

How will you maximise the outputs of your work?

The work from this project licence will be disseminated between the MINIMISE project members at regular intervals (biannually). New knowledge will be showcased at international conferences with a special interest in plastic pollution (e.g., Society of Environmental Toxicology and Chemistry and International Conference on Environmental



Effects of Nanoparticles and Nanomaterials). The data will be compiled into manuscripts to be submitted to high impact journals for peer review, with each of the three objectives likely to produce at least one publication (>3 total).

Species and numbers of animals expected to be used

- Zebra fish: 2000
- 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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Fish will be exposed to novel plastic particulates, with or without adsorbed co-contaminants, through the water or diet to assess their uptake and toxicity. The fish will be exposed via the food or water for a number of weeks, with the only change to their normal husbandry being the addition of plastic materials at sub-lethal concentrations. We are not expecting any lethal toxicity from these materials.

The maximum exposure a fish will endure is 4 weeks. Each fish will undergo one procedure only before being killed by a schedule 1 approved method. The fish will then be dissected to remove the tissues for assessment of plastic and/or chemical uptake or toxicological assays.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We do not expect there to be any adverse effects to the animals. However, when using novel materials that have not been tested before, it is difficult to definitively say. Although not expected, some of the particles could cause transient gill or gut irritation at the start of the exposure.

Salmonids could display adverse effects including vomiting, loss of equilibrium, and changes in behavior or lesions. Zebrafish could display adverse effects including abnormal swimming orientation, lesions, scoliosis, scale and pigmentation loss, or become severely egg-bound. These will be recognized by generating species specific scoring index's, with fish monitored 3 times per day to check for development of clinical signs. It is expected that most of these symptoms would be transient, but if they persist for more than 24 h the fish will be removed from the experiment and killed immediately using a schedule 1 approved method.

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

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



As we do not expect any adverse effects, but cannot exclude them, we have selected the severity category to be Moderate. As we cannot currently say if a particle characteristic is toxic, the chance of them feeling any pain or distress could be around 50%.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Our aim is to understand the uptake and toxicity of plastic particulates (and their co-contaminants) across the gills and gut of fish. The factors that determine the amount of chemical that stays in the body of a fish is dependent on the adsorption across the gill or gut, its distribution around the body through the blood supply, any metabolism, and any subsequent excretion that occurs from the body (e.g., from the liver or kidneys). Therefore, to truly understand how these plastic particles affect fish, we need live fish to be used and exposed via the gill or gut. There is no suitable non-protected animal that would be a viable substitute.

What was your strategy for searching for non-animal alternatives?

As a vertebrate alternative, we have considered the use of *in vitro* gut and gill cell cultures systems, as well as *ex vivo* gut sac techniques.

As an aquatic organism alternative, we considered the use of invertebrates.

Why were they not suitable?

The media used to maintain the cells/tissues alive uses quite high concentrations of salts, which is known to affect the particle stability. The main problem with this is it can cause particles to aggregate and settle on the cells/tissue. This can lead to higher dose than would be expected when exposing fish to the chemicals. For example, using metallic nanoparticles, the tissue level techniques (e.g., the gut sac) suggests up to 20% of the exposure dose can be taken up by the intestine, whereas in exposed whole fish it is around 3% or less. Therefore, to ensure our results are realistic to exposure in the environment, live fish need to be used.

The use of invertebrates are also not appropriate to replace fish studies. Fish have a closed circulatory system (i.e., the blood supply) which can distribute chemicals around the body once they have passed either the gill or gut. In contrast, invertebrates have many tissues exposed to the surrounding water which means it is difficult to establish the relative uptake and toxicity from plastics in the water versus the food. Therefore, to understand how different exposure pathways affect the uptake and toxicity in organisms, fish need to be used.

Reduction



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

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

The experimental design is for each treatment to occur in triplicate, with the tank as the recognized replicate. This triplicated experimental design has been scrutinized in peer review and is well established for experimental work with fish. To determine the total number of fish needed in an experiment, Mead's resource equation will be used.

For assessment of uptake, power calculations show $n = 9/\text{treatment}$ need to be sampled. In a triplicated experimental design, this means $n = 3/\text{tank}/\text{time point}$. The longest planned study is 8 weeks (4 weeks uptake and 4 week depuration), with a sampling rate for this analysis at once per week. Therefore, a maximum of 8 time points in one study equates to 24 fish per tank.

For toxicological assays, the same power calculation applies. However, a maximum of 4 sample points will be taken during the experiment. Therefore the total fish needed for toxicological endpoints are 12.

In order to ensure social hierarchies are stable until the end of the experiment, and additional 4 fish will be used.

Therefore the overall number of fish per tank is up to 40 fish (120 per treatment) in each experiment.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

To determine the total number of fish needed in an experiment to allow for statistical differences to be achieved between treatments, Mead's resource equation and power analysis will be used. Power analysis has given estimates of 8-9 fish per treatment ($n = 3/\text{tank}/\text{time point}/\text{end point}$). The end points are two-fold: accumulation and toxicity (maximum total of 6 fish/tank/time point). For the toxicity end points (histology, biochemistry and molecular biology), multiple data points can be achieved by sub-sampling tissues from the same fish, which further reduces the number of animals involved.

To ensure robust data and to avoid repeating experiments (i.e., reducing the animals used), various sources of variation are being reduced. Many of the sources of variation are known due to previous experimental work being conducted in these systems. The tanks will be randomly allocated to account for any variation in the environment exterior to the tank (e.g., nearby cooling units). Previously, we have blinded treatments where appropriate (e.g., histological analysis) to prevent biases in analysis. An additional source of variability is dissecting whole organs (e.g., separation of the gallbladder from the liver) at sampling points. To control variability from sampling, the minimum amount of researchers dissecting fish on a given sampling day will be used, with only individuals with previous experience being involved (e.g., the NACWO). Also, dissecting tools will be acid washed prior to sampling to avoid contamination, as well as being washed between treatments.



What other measures apart from good experimental design will you use to minimise numbers?

To determine the total number of fish needed in an experiment to allow for statistical differences to be achieved between treatments, Mead's resource equation and power analysis will be used. Power analysis has given estimates of 8-9 fish per treatment ($n = 3/\text{tank}/\text{time point}/\text{end point}$). The end points are two-fold: accumulation and toxicity (maximum total of 6 fish/tank/time point). For the toxicity end points (histology, biochemistry and molecular biology), multiple data points can be achieved by sub-sampling tissues from the same fish, which further reduces the number of animals involved.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We are exposing fish via the water or gut to a range of plastic particulates (and/or co-contaminants) for up to 4 weeks. Salmonids could display symptoms including vomiting, loss of equilibrium, and changes in behavior or lesions. Zebrafish could display signs of abnormal swimming orientation, lesions, scoliosis, scale and pigmentation loss, or become severely egg-bound. These will be recognized by generating species specific scoring index's, with fish monitored 3 times per day to check for development. It is expected that most of these symptoms would be transient, but if they persist the fish will be removed from the experiment and killed using a schedule 1 approved method. The exposure concentrations are as close to environmentally realistic as possible, reducing the chance of pain, suffering, distress or lasting harm. Also, the delivery of the plastic particulates is in the same manner as those that fish are naturally being exposed to in the environment. As a result, we do not expect the fish to show clinical signs of exposure. The generated data is only satisfactory so long as the fish is healthy and not showing signs of ill health.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Part of this project will use less sentient animals; the OECD 236 zebrafish embryo toxicity tests (OECD 2013) will be used to screen novel plastic particulates where data for sub-lethal thresholds do not exist. These will then be used to inform suitable exposure concentrations. Also, where existing data to inform the exposure concentrations do not exist, a small pilot study will be conducted which will involve escalating dose design.

A fundamental part of uptake of particulate material is crossing a biological barrier to the blood supply for systemic circulation around the body (i.e., a closed circulatory system). Crossing of particulate material through this externally facing membrane is also a vertebrate specific anatomy, as invertebrates have open circulatory systems whereby the exposure media comes into contact with multiple tissues at once (e.g., mussels). While



this can give a specific affinity for a tissue or tissue surface (e.g., digestive gland), it does not allow the rate limiting factor of uptake to be overcome – usually the basolateral membrane of the gill or gut epithelium – which will inherently affect the rate of uptake. Also, it allows sampling of internal organs which have not come into contact with the water or food so we can accurately assess what has been accumulated and not surface bound to the exposed tissues.

We cannot use terminally anesthetized fish as this would interfere with the overall uptake and excretion process, and hence the aim of this project.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals will receive visual health checks 3 times per day. The water quality will be checked daily and reasonable adjustments will be made in case water quality parameters are unsatisfactory (e.g., increased flow rate/water changes). Any animals showing adverse effects will be removed from the experiment, so long as they are not thought to be treatment related. If clinical signs arise then the NACWO and/or the NVS will be contacted regarding the longevity of the experiment.

As the use of vertebrates is needed, part of our refinement is using fish and exposing them to sub-lethal concentrations, making them unlikely to be aware they are being exposed. Also, exposure concentrations of novel plastic particulates will be screened using the OECD 236 zebrafish embryo test (OECD 2013) to help determine sub-lethal concentrations.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow the OECD 305 test guidelines. Also, where we do not have established data for threshold levels, small pilot studies will be used (e.g., escalating dose designs). Such experiments will start with a reduced number of experimental tanks (e.g., 2).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

This will be approached by:

Regular checks on the NC3R's website.

Contact with the Named Information Officer for any current updates.

Explain the choice of species and the related life stages

Salmonids and zebrafish are widely used in ecotoxicological studies, ensuring the plastic particulates we assess can be compared to a variety of other chemicals. Salmonids will be used for several reasons:

Their large size allows relative ease for sampling.

A large body of literature and previous studies exist on them, allowing for material comparison.



Part of the life cycle being in the ocean, it increases the degree of extrapolation from freshwater to seawater species.

They are commercially important species.

The zebrafish will also be used for uptake and toxicity studies. The main reasons for this includes:

Fully characterized genome and proteome for molecular studies of sub-lethal toxicity.

The small bodies will allow waterborne radiolabelled experiments to occur within the ionizing radiation regulations (IRR17) that larger fish make problematic for long period.

Environmental sampling of the gut contents of adult fish shows the presence of plastic particles. Therefore, to understand the effects these particles are having in the environment, we need to use the appropriate life cycle stage of the fish.





35. Developing and evaluating imaging biomarkers in oncology

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

There are two parallel aims for the project.

To develop new imaging biomarkers for use in REDACTED studies. This work focuses around the technical and biological validation of imaging biomarkers (a characteristic by which a particular pathological status can be measured) that are not yet ready for decision-making.

To use established imaging biomarkers to assess therapies. This work focuses on using imaging biomarkers for decision-making.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 imaging involves many approaches that include magnetic resonance imaging (MRI), computed tomography (CT), ultrasonography, positron emission tomography (PET) and single-photon emission computed tomography (SPECT). Imaging biomarkers, from these technologies provide a crucial role in the assessment of REDACTED patients, from early detection and diagnosis through to response assessment and prognostic and predictive evaluation.

As new therapies are developed, that target different aspects of REDACTED, so too there is a need to develop appropriate new biomarkers to detect, diagnose and monitor the new therapies. This need applies a) for both primary and metastatic disease; b) across a wide range of tumour types (e.g. Lung, Breast, Colorectal, Prostate, Gliomas, Renal, Gynae tract, Melanoma and others); and c) for a wide variety of targets (e.g. specific pathways, general microenvironment readouts such as hypoxia or immune cell populations).

As part of the above, new imaging biomarkers must be developed and then applied to studies of new targets related to the hallmarks of REDACTED. Developing biomarkers is a very complicated process that requires phantom, preclinical model and clinical studies. Similarly, evaluating new therapies requires preclinical model and clinical studies. This project licence is focused on developing and evaluating new imaging biomarkers in studies of various REDACTEDs.

Aim 1:

Many new imaging biomarkers are in development, but only a small minority make it through to clinical adoption. This is due to a failure to prove or validate the imaging biomarkers.

The need to have a rigorous framework of imaging biomarker validation is now recognised by the imaging science community. One key step is performing technical and biological validation steps in various preclinical REDACTED models, which include; Lung, Breast, Colorectal, Prostate, Glioma and Melanoma, as well as other models. In particular, this need has been articulated in an international consensus report, led by the PPL applicant.

Validation occurs using a variety of models, with choice of model determined by the biomarker in question. For example, imaging biomarkers of hypoxia (low oxygen content) should be developed in models that span a range of tumour types and have a range of values of hypoxic fraction. Similar principles apply to developing imaging biomarkers for use in immune-oncology, but the particular choice of models and the host mouse will differ since other biological systems will be more appropriate (e.g. syngeneic mouse v immunocompromised). Initial work will often begin in relatively simple models, but there is need to replicate key findings in more clinically relevant models (e.g. patient derived tumour implant models (PDX), metastatic lesions etc).

Aim 2:

Imaging offers the ability to perform real time and dynamic in vivo whole tumour tracking of response to therapy. This makes imaging an attractive tool in the assessment of new therapies or new combinations of therapies, since serial monitoring can provide information on the timing of anti-tumour efficacy for the primary tumour, secondary and metastatic disease across various modalities.

Again, choices of model will vary for each type of therapy under investigation, and a range of models starting from simple and moving to more complex (e.g. PDX, metastatic) should be used.



Furthermore, imaging may enable reduction of use of mice by providing methods that are more sensitive to detecting treatment effect. The PPL applicant's group have already begun to investigate how imaging may enhance experimental design by reduction of use.

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

The primary output of this project will be a substantial amount of data – information that advances our understanding of:

which imaging biomarkers should be selected for further application in preclinical studies/clinical studies, and;

mechanistic knowledge of therapies, based in part from established and new imaging biomarkers.

Outputs will be publication in peer-reviewed journals and presentations at scientific conferences and meetings.

It is anticipated that data will be used for subsequent grant applications.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Other preclinical scientists will benefit since the validation work (aim 1) will let them make informed decisions about which imaging biomarkers are accurate, precise and biologically relevant, for them to use in their own studies.

Clinical trialists will benefit since the translational work (aim 2) will let them make informed decisions about which imaging biomarkers are accurate, precise and biologically relevant, for them to use in early phase clinical studies.

How will you maximise the outputs of your work?

As well as publication (detailed above), we will aim to develop new collaborations. This is something that the group is doing already (with collaborators in the UK and other countries). We will also ensure that new knowledge is disseminated within the UK via education workshops that are run by the applicant.

Species and numbers of animals expected to be used

- Mice: 4800

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



Most mice used in our project licence will undergo testing of drug regimens to confirm they are tolerable over a period of time ~25 days. New mice will then be injected with tumour cells of various REDACTED types to emulate the REDACTEDs patients display in the clinic.

Similar to patients we will monitor the tumour growth and dose them with relevant rationale for REDACTED treatment with a series of drugs or combination of these. These treatments may either be potential novel therapeutic agents, existing clinical agents or placebo administered by a variety of routes, but usually either by mouth, or by injection either under the skin or into the abdomen to study the effects on tumour growth and / or tumour composition. The mice will also have blood samples taken either from the tail vein or by sampling from a heart chamber under anaesthesia.

In some cases the treatment will include radiotherapy in combination to chemotherapy for specific REDACTED types. Other experiments will also implement a combination of chemo/radiotherapy with immunological agents to augment the therapeutic benefit.

Along these treatment trials we will also aim to imagine the mice under very similar conditions to patients using equipment like MR, CT and PET scanner to visualise the intravital details of the tumour and biomarker of response. To simulate the patients with advance disease, in a large portion of the experiments we will excise the primary tumour and wait to monitor the growth of distant REDACTEDs (metastasis) which will then be treated and imaged as the primary tumour.

On some occasions it will be necessary to condition the mice for receipt of tumour cells by either modifying the diet or water (in the case of hormone positive breast REDACTEDs), administration of immune cells or radiation to prepare the host for tumour implantation.

Mice may be studied for up to 200 days after a period of therapeutic agent treatment for tumour growth to track long lasting benefit and imaged to confirm this. Fast growing tumours will always be monitored daily.

Mice will be group housed in ventilated cages which have their environment enhanced with items such as tunnels, houses, nesting material and gnawing blocks.

At the end of any protocol mice will be humanely killed by.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The greatest impact of the work carried on the mice will be primarily be the tumour growth and implantation procedures. Therefore it is possible that the tumour growth might affect normal physiological functions (such as locomotion or breathing depth) however, mice will be observed daily and any side effect that cannot be managed satisfactorily, e.g. loss of body weight by dietary supplementation with seeds, will be killed humanely.

Injections for labelling agents, tumour cells or drug delivery would only cause very transient minimal pain.

After surgical procedures for (tumour implants or removals) we will monitor mice for signs of pain and administer effective pain relief for as long as it is required.



The impact from the imaging sessions on the mice will only be limited to the insertion of the cannulas where required and the anaesthesia itself.

As with all procedures animal welfare will be first consideration and any harm or discomfort will be addressed leading to a termination the mice under schedule 1 methods.

The same will apply to the therapeutics intervention where the required pilot and toxicity studies will be done prior any large experiment to test tolerability of any compound or combination used along with the imaging.

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

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

The vast majority of mice are only expected to experience the pain of repeated (daily) injections of therapeutic agents. We will aim to utilise the least stressful route, of administration wherever possible, such as oral gavage. All therapeutics regimes will be evaluated to minimise any induction of weight loss during treatments.

In the case sub cutaneous tumour growth we will monitor the progression on a regular basis to ensure that the scientific and humane end-points are followed.

For orthotopic models we will use palpation and intravital imaging to tract tumour growth and this will mean repeated anaesthesia for the purposes of imaging the internal tumours.

Therefore we foresee to have most of our mice, approximately 70% experience severities closer to the lighter side of a moderate severity band.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

While valuable studies of human REDACTED are performed using tumour material and cell lines derived from both mice and human samples, the mechanistic understanding of REDACTED pathogenesis and the biological & physical parameters of imaging requires use of living animals.

In particular, REDACTED development and spread involves a plethora of interactions between REDACTED cells and their surrounding host and their behaviour is governed by multiple signals originating from both their immediate neighbours and from distant tissues.

The REDACTED models implemented in our work, aim to closely mimic their human counterparts, and have the potential to test the effectiveness of novel REDACTED



therapeutics along with the evaluation of imaging biomarkers. This cannot be replaced by in vitro studies which remain far less complex than a murine host.

What was your strategy for searching for non-animal alternatives?

We will use a variety of in vitro / ex vivo approaches to investigate how different drugs and therapeutic combinations can inhibit REDACTED growth prior to undertaking in vivo studies.

In addition, we will use molecular pathology (e.g. immunohistochemistry) to substantiate findings from our in vitro / ex vivo models in human tumour samples from clinical trials.

Other methods to be utilized include established cell biology techniques to measure; cell proliferation, survival, migration, and invasion. Where required we will also use biochemical and molecular biology techniques such as western blotting, enzymatic assays, proteomics, RT-PCR to study molecular pathways.

Why were they not suitable?

The study of cells in culture (in vitro) provides us with clues on the mechanisms of cellular processes in a simple and valuable context, which allows the establishment of hypotheses regarding the function of cells in a living animal. However, these systems do not recapitulate the complex cellular interactions described above.

Reduction

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

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

Most of the therapeutic regimes are established as they are part of clinical practice and clinical trials. In the case of novel agents or new combination (chemo/radio) we will in vitro methods to guide doses and combination rationale, this in turn will limit the number of animals required for the in vivo investigation stage.

The overall aim will be to generate models whereby a measurable effect e.g. reduction in tumour volume following treatment combinations can be clearly determined using a minimal number of animals. In general, using 80% power, 95% confidence and 20% practical difference, group sizes of 6- 10 animals per experiment should suffice. Pilot studies will be performed if applicable and, after analysis of the results using our published methodology, group sizes for subsequent experiments will be determined based upon these data.

We will be conducting and recording our experiments to be able to publish our results following the ARRIVE guidelines [<https://www.nc3rs.org.uk/arrive-guidelines>] and will use randomisation, blinding etc. where appropriate so as to minimise biases.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?



We will discuss studies and powering of them, based upon data from pilot studies with local statisticians. Furthermore, we will discuss studies and powering of them, based upon data from pilot and similar archive studies plus in collaboration with a biostatisticians to cover calculating group size and ensuring statistical robustness. Furthermore, additional resources may be used to aid experimental design such as the NC3Rs experimental design assistant tool (EDA).

(<https://www.nc3rs.org.uk/experimental-design-assistant-eda>).

What other measures apart from good experimental design will you use to minimise numbers?

Pilot studies will be performed where required and after analysis of the results, group sizes for subsequent experiments will be determined based upon these 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use relevant preclinical REDACTED models that display true clinical features, will also have realistic clinical readouts like perfusion-weighted imaging (PWI), and diffusion-weighted imaging (DWI) that can inform biomarker derivation. This raw data will be used in a series of computer modelling to allow gaining the most insight from the captured data per experiment.

Past in vivo experience will be used to guide experiments to help to minimize potential suffering by regular monitoring of tumour size and potential impact on the general health status of the mice. For any new procedure, we will seek expert advice from REDACTED staff (e.g. NVS, NACWO and HOI) follow the most refined techniques available.

For any inoculation/transplantation procedure we will follow the route that causes the minimal burden on the animal's well-being.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice are more comparable to humans than less sentient model systems (fish, invertebrates) in development, physiology, and genetic complexity. In mice we can model not only anti-tumour therapy effects but also drug metabolisms (absorption and excretion) profiles

Non-protected species and less sentient species do not have a lung or the complex signalling required for development of the lung or mammary REDACTEDs.



In addition the imaging equipment is primarily designed to image live mice. Where possible we can introduce using organoids to some aspects of the project to inform future in vivo work.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

To refine the type and number of procedures we will carry out an assessment on the data from the sizes of superficial tumours captured in our studies to derive a table where we can quantify the ideal tumour volume needed for imaging.

The total tumour burden should not exceed 1500 mm³, and volumes will be monitored closely, in the case of tumour approximately ~800mm³ of the maximum permissible volume, it will be measured more frequently.

During tumour growth, external tumours will be monitored for progression from local inflammation/irritation/pinpoint scabbing to an ulcerated state, depending on the tumour type (e.g. melanoma or breast) and especially after radiotherapy. Animals with ulcerated tumours will be cared for according to the best practice at REDACTED.

Where blood sampling is required, we will use microsampling. These samples will be taken according to good practice [Morton et al., *Lab Animals*, 35(1): 1-41 (2001); Workman P, et al. *British Journal of REDACTED*, 102:1555-77 (2010)] using the least severe technique appropriate for both the strain of mouse and experimental requirement (e.g. venepuncture of a superficial blood vessel) with sample volumes being kept to a minimum and not exceeding published limits (10% at any one time and 15% over a 28 day period) and allowing appropriate recovery periods.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Throughout our work we will refer, consult and adhere to the guidelines and practices discussed in a series of key publications.

Miller AL, Leach MC. The Mouse Grimace Scale: A Clinically Useful Tool? *PLoS One*. 2015;10(9):e0136000.

Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, et al. Guidelines for the welfare and use of animals in REDACTED research. *Br J REDACTED*. 2010;102(11):1555-77.

Morton DB, Jennings M, Buckwell A, Ewbank R, Godfrey C, Holgate B, et al. Refining procedures for the administration of substances. *Lab Anim*. 2001;35(1):1-41.

Ullman-Cullere MH, Foltz CJ. Body condition scoring: a rapid and accurate method for assessing health status in mice. *Lab Anim Sci*. 1999;49(3):319-23.

How will you ensure you continue to use the most refined methods during the lifetime of this project?



One of the team working on this PPL sits on the 3Rs Network committee at REDACTED where 3R initiatives are discussed and spread. Also by reviewing and reading regular newsletters from the NC3Rs.

Explain the choice of species and the related life stages

Mice are the ideal experimental host not only due to their size and quick life cycles but because their genetic, biological and behaviour characteristics closely resemble those of humans, and many symptoms of human conditions can be replicated in mice to a high degree of similarity. We use mice over 4 wks of age, as they are considered to be of adult age to sustain tumour growth and can tolerate a large degree of therapeutic interventions.



36. Developing fertility control as a management tool for REDACTED

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Animal types	Life stages
Rats	adult, pregnant, aged
REDACTED	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 is the aim of this project?

This project aims to produce an effective fertility control system suitable for use with REDACTED in the field.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 REDACTED has become widespread across much of England and Wales and is continuing its expansion northward into Scotland [Mammal Society, 2018]. Consequently the degree and extent of the problems caused by REDACTED have increased, which include threats to forestry and biodiversity. Importantly the continued spread of the



REDACTED also threatens the remnant populations of the endangered REDACTED where they still occur across GB.

The only legal and effective tools to manage populations of REDACTED are lethal methods (shooting and live trapping with dispatch). Both are inefficient (especially at low densities of REDACTED) and unsuitable in many areas where REDACTED are common (e.g. urban and sub-urban landscapes).

Modelling work has indicated the potential value of fertility control in helping manage problems caused by REDACTED when integrated into a coordinated landscape-scale control program alongside other (lethal) methods. Fertility control may permit the cost-effective control of REDACTED populations at low- densities, as well as the management of populations where lethal methods are socially unacceptable (e.g. urban areas) or technically unfeasible. The most practicable approach to delivering fertility control for REDACTED is using oral contraceptive delivered in baits to free-ranging REDACTED. However this requires detailed studies to:

identify and characterize fertility control agents which are suitable for REDACTED (e.g. safe and with promising properties for fertility control)

develop and optimize the most practicable and effective formulation for a self-dosing deployment. For example the oral delivery of an agent may include research into the optimum combination of other ingredients such as adjuvants, additives, baits, preservatives etc.

validate the efficacy and ensure the humaneness of the most promising contraceptive in a proof of concept experiment

quantify key aspects of REDACTED behaviour and ecology in the field

to support the design and deployment of the field-based devices used to administer the contraceptive

underpin mathematical models which will be used to provide replacement and reduction for this program of work

enhance the benefit of the research by demonstrating the utility of the work to a wider audience and practitioners

References

Mammal Society (2018) A Review of the Population and Conservation Status of British Mammals (JP025)

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

We will have developed (a) formulation(s) with the necessary properties to contribute to an effective, humane and practicable fertility control system (e.g. enhanced fertility control, easy delivery) and selected the best candidate contraceptive for a proof of concept experiment using a breeding colony of REDACTED maintained for the purpose.

We will have undertaken a captive experiment demonstrating the efficacy and humaneness of one or more formulations.



We will have developed devices and deployment strategies suitable for use with the most effective and practicable contraceptive.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

If successful we will have developed a system comprised of tools (fertility control agent, formulation, delivery device and deployment strategy informed through mathematical modelling) which can be adopted by practitioners in the field and used as part of a comprehensive REDACTED control strategy. This should bring benefits to the economy (protecting crop trees), biodiversity (where trees and birds are threatened by REDACTED), as well as supporting the conservation of the REDACTED. This work also acts as the foundation stone upon which further development of any of these tools may produce enhanced benefits in the future (e.g. application to other species and the solution to other human- wildlife conflicts).

Even if only partially successful, we will have driven forward the development of science in one or more of these component tools and contributed to a number of areas of basic science. Particularly the understanding of the reproductive physiology of REDACTED, their ecology and behaviour in the field, as well as some of the basic principles of developing immunologic approaches for wildlife applications which may bring benefits in other areas or for other species.

How will you maximise the outputs of your work?

Elements of the work will be published in the peer-reviewed literature and will be disseminated at technical and scientific conferences as well as meetings of practitioners and stakeholders.

REDACTED keen to see any benefits accruing from this work translated into practical benefits as quickly as possible, and communications plans are already being discussed.

Species and numbers of animals expected to be used

- Rats: 200
- Other rodents: No answer provided

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

REDACTED will be used in two types of study. In the field animals will be marked using PIT tags but will otherwise be unaffected by our work and will be released to continue their lives in the wild; we will use anaesthesia to make sure that this work is as refined as possible. Some REDACTED will be brought into a specialist wildlife facility in the laboratory to participate in experiments to develop a contraceptive. This facility is set up to



care for the REDACTED as well as possible. Typically these animals will be administered with a fertility control agent or formulation, and their responses measured using blood samples or non-invasive techniques (e.g. ultrasound) which require the REDACTED to be handled (refined using anaesthesia). Dosing REDACTED may involve injections or other regulated methods but candidate contraceptives are expected to often be self-administered in food or drink.

Rat (used as a model species) will be administered with a fertility control agent or formulation, and their responses measured using blood samples. This will involve injections, other regulated methods (gavage or oropharyngeal dosing) or be self-administered in food or drink.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

As our goal is the production of a fertility control system suitable for use in the field, we are working towards contraceptives free of harmful side effects to the REDACTED, or harmful effects on the environment. Most of the laboratory based research we will undertake will be using substances which are not intended to produce any harms to REDACTED, and are intended only to reduce their reproductive capacity. Developing the contraceptive will require some REDACTED to be injected, though any harmful effects these injections have are expected to be mild and short-lived. More generally, many REDACTED will need to provide periodic blood samples or be handled and observed closely (e.g. ultrasound examinations). These are best done by anaesthetising the animal, which reduces the stress it experiences as well as permitting work to proceed calmly and efficiently to produce the very best science. A small number of animals might react poorly to some treatments but close observation and rapid interventions to limit harms will ensure that suffering is minor and brief.

Some rats will be injected with a fertility control agent where they may suffer some mild and transient pain that is not expected to last more than 72 hours. There may be an injection site reaction manifesting as sterile granulomas but causing no pain to the rats. Rats administered the fertility control agent via oropharyngeal dosing may experience some mild respiratory distress if the agent is viscous although this should be overcome by splitting the treatment into sub doses.

The REDACTED we study in the field should experience no significant adverse effects. Indeed competent staff will undertake a number of checks to ensure that the animal is fit for release, free of unintended injury, and able to continue living in the wild without detriment.

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

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

Most animals (estimated at 90%) will experience a mild severity. No animal will exceed a moderate severity.

What will happen to the animals at the end of the study?

- 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?

Developing a contraceptive for REDACTED and a delivery device for its use in the field, as well as a strategy for deploying it to manage the environmental and economic impact by REDACTED cannot be done without research using animals. Whilst research discovering and describing new agents which can produce fertility control in REDACTED can use other rodent species as models for some aspects of the work (i.e. laboratory rats), key experiments still require us to study REDACTED to check and confirm our research. Using REDACTED is essential to demonstrate that our contraceptive is safe for REDACTED and effective. In addition, the practicability and cost-effectiveness of the system also depends on the performance of the devices we develop and how wild free-living REDACTED behave around them, requiring the study of wild REDACTED in the field.

However computer modelling study is running in parallel with the animal study to refine parts of the field trials.

What was your strategy for searching for non-animal alternatives?

REDACTED are unusual study species. Not much is known of their reproductive physiology and less is known of how to prevent their reproduction. As such non-animal methods known to robustly replicate their reproductive physiology are not available; requiring the use of animals to undertake the basic science as well as research how to control their fertility.

However, we do use mathematical modelling to identify when experimental work with animals has produced a contraceptive sufficiently good to use in the field. The modelling takes the empirical estimates we produce as part of this study (both of reproductive inhibition in captivity and behaviour in the field) and combines these to predict the efficiency of our fertility control system at the population scale across realistic simulations of real-world landscapes. This avoids undertaking extensive and lengthy study of animals in the field to provide the same evidence.

Why were they not suitable?

There are no non-animal alternatives to study the details of the reproductive physiology of REDACTED, its inhibition using previously unidentified contraceptives, or describe for the first time the behaviour and ecology of REDACTED in the field when presented with our self-dosing devices.

Reduction

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



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

We have used our previous experience of identifying and characterising fertility control agents or contraceptives in other species to estimate how many animals we might use to achieve our goal of producing a fertility control system for REDACTED.

Where possible the most efficient statistical designs will be used (e.g. factorial designs), though where the constraints of animal housing (confounded blocking) or the measurement of unwanted interactions precludes this type of approach, power calculations will be used where appropriate to determine animal numbers required (usually groups of 7 to 10 animals). Thus where laboratory rats will be used as a model species smaller groups may be sufficient; and the greater variability expected in wild caught REDACTED suggest the slightly larger sample size. Wherever possible work will be planned to optimise the use of animals in control groups. Excepting specific study types (e.g pilot studies) many experiments will follow a pattern comparing a number of treatment groups with a control and use around 30 animals.

Laboratory rats are useful for studying some of the basic science around REDACTED reproduction and fertility control, or the initial work in describing the potential for a new substance or its safety to REDACTED. Whilst we expect most studies to be relatively small (use around 30 animals) we cannot know how many studies we may need to achieve our aims and have specified a number similar to that used on previous similar licences. However, we will always need to check our results in experiments using REDACTED to make sure that our understanding is sound and expect to use a similar number of REDACTED in laboratory studies (300 animals).

Our plan includes a proof of concept study where we demonstrate the safety and efficacy of our chosen contraceptive, and this needs to use the REDACTED in our breeding colony. Our breeding colony is currently established at 72 REDACTED. It is likely that some animals will be lost to old age through the course of study and need replacing. Thought this number is unlikely to be large it is difficult in this application to estimate accurately how many additional animals may be needed to maintain the colony in anticipation of our final proof of concept study. We have estimated the number needed here will not exceed 150 REDACTED. An additional 25 animals might be needed to just produce the basic descriptions of REDACTED reproductive physiology which we think will help us to produce better science as well as help us to design shorter and more refined studies.

About half of the REDACTED we might use will be animals which have marked with a PIT tag and then released back into the wild to continue their life there (approximately 300 REDACTED). The number of REDACTED we actually use in field experiments may be much smaller than this as the number present in the woodlands where we work is always uncertain (woodland size and character affect the number of REDACTED present, as well as the season of study). We have anticipated two separate studies, each comparing three woodlands, each hosting about 50 marked REDACTED.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

All study designs will be subject to review by the PPLh (not the scientist leading the work), a statistician and the AWERB (including an independent statistician), all with a view to ensuring that studies are appropriately but not excessively powered to achieve their aims.



Where the evidence required to design appropriately powered studies is unavailable (i.e. variation in dose-response to an agent), expert opinion and experience will be used to arrive at a suitably robust design for small pilot studies, which themselves will inform the design of larger experiments if these are required.

What other measures apart from good experimental design will you use to minimise numbers?

Our use of mathematical modelling permits us to potentially achieve a substantial reduction in the number of animals required to produce a fertility control system in REDACTED. Modelling allows us to identify, at the earliest moment when the development of contraceptive formulations and self-dosing devices can stop, and when their combination produces a fit for purpose system.

Useful fertility control systems do not have to be perfect, i.e. produce permanent, 100% infertility in 100% of REDACTED. Modelling has demonstrated how contraceptives delivered to sufficient REDACTED may still a cost-effective method to reduce the number of REDACTED at the landscape scale. Trade-offs exist in how the properties of the agent, its formulation and its deployment in self-dosing devices can be combined to achieve a useful reduction in the populations of REDACTED based on fertility control. Modelling will be used throughout the progress of this work to identify when the development and optimisation of agents or formulations (both of which use animals) can be stopped in favour of enhancing the design of the self-dosing delivery devices or the schedule of deployment.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Much of our work will require the use of live REDACTED, as it only in this model that the specific reproductive physiology we need to study and modify is expressed. This is also the target species for our overall aim. However, work on REDACTED in the laboratory requires them to be brought into captivity, as well as being subject to the limitations of working with a wild animal (distress during routine handling, infrequent and seasonal breeding attempts), and a more tractable model for some of the basic science is available in the laboratory rat. Both are rodents and are likely to share similarities in some physiological processes. As laboratory rats are much more suited to laboratory based research on fertility control, being comfortable in captivity and constantly undergoing reproductive cycles, their use for some of the work is considered a substantial refinement.

Part of our work involves the study of REDACTED in the wild. Our use of anaesthesia to refine the experience of REDACTED as they are marked with PIT tags, and their prompt release back to the wild represents the most refined approach we can achieve. We will also ensure injuries and distress are reduced by using best practice in trapping (covering traps, regular checks etc).



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

There are no known models for the physiology of REDACTED that might be considered less sentient. Laboratory rats are no less sentient than REDACTED but are at least habituated to husbandry in the laboratory and are more comfortable being handled where this is required for the work.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The work applies refinement and continuous improvement to all elements of the work in this plan.

Specifically the care and husbandry of the REDACTED in captivity is under constant review by a specially convened species specialist team, which includes the NVS, NACWO, REDACTED scientists and REDACTED technicians who know their animals well. Refinements instituted by this team include minimising unnecessary contact with humans (e.g. remote observation 24/7 using infra-red video recording) and establishing strong routines (times, staff members) to reduce stress to the REDACTED. In addition, captive REDACTED have an evolved program of rolling enrichment, for both the facilities within their home pens (branches, hiding places, substrate) as well as feeding (food and presentation of food). This enrichment is considered carefully, as too much change is now thought to be stressful for the animals.

Additionally, most scientific interventions are refined using anaesthesia, which minimises the stress caused to the animal, permits the collection of multiple samples or observations through a single intervention without additional stress to the animals, as well as offering operators the opportunity to achieve protocol steps safely and securely, producing the best care for the animal and best science possible.

The breeding colony of REDACTED will be primarily monitored using remote CCTV so that there will be less disturbance and to minimise stress. Only if the REDACTED can not be observed via the CCTV or if there is cause for concern will they be psychically checked.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The two collaborating organisations have unparalleled experience at housing and breeding REDACTED in captivity. This success is produced in part by the unique research facility where the REDACTED are housed and provides the space and conditions for their optimum care; and also by constant attention to the refinement of regulated work involving captive REDACTED and enrichment of their care and husbandry. As part of both of these processes a REDACTED specialist group oversees the constant review of the care and use of REDACTED to ensure the highest standards are met.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

This plan is delivered as a collaboration between two organisations, both of which are committed to ensuring the most refined regulated work possible. The joint AWERB



considers the 3Rs of all studies put forward and has expertise from a range of wildlife scientists, vets and statisticians. Both organisations employ staff who keep abreast of the latest advances in the care and research of laboratory rats. Both organisations also send staff to other organisations caring for REDACTED, or studying them, to bring back new ideas to further improve our culture of care for than animals in captivity and improve their experience during the research.

Explain the choice of species and the related life stages

Only the study of REDACTED allows us to develop and demonstrate an effective and benign self- dosing fertility control system intended to reduce their populations in the field. The behaviour of free living REDACTED can only be studied in the field. Some animals will also be brought into captivity to permit the robust science and inference necessary to develop and test our contraceptives.

Where laboratory rats can be used as a model species, especially in the initial evaluation of fertility control agents or formulations, they will be used as they represent an effective replacement and one more suited to the laboratory environment and their reproductive cycle being shorter means that the studies can be quicker.





37. Developing Genetic Therapies for Respiratory 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

No answer provided

Animal types	Life stages
Mice	juvenile, adult, 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 is the aim of this project?

We aim to develop genetic and cell therapies, e.g., by gene replacement, gene editing, gene silencing, or by engraftment of cells into the lung, for the treatment of inherited diseases such as cystic fibrosis and primary ciliary dyskinesia, or infectious diseases such as Covid-19 affecting the lung.

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

Why is it important to undertake this work?

There are several inherited diseases that specifically affect the lung including cystic fibrosis, primary ciliary dyskinesia (PCD), surfactant protein deficiencies, alpha1-antitrypsin deficiency and others, that are currently untreatable. CF affects about 10,000 people in the UK while PCD affects about 1,000. CF patients on average are dying in their thirties although there are some new drugs available for about half of patients but the other half still requires new treatment alternatives. PCD is not quite so serious but still can



require lung transplantation in serious cases. Genetic approaches offer the possibility of new therapies for these diseases. While preliminary work can be performed in human cultured cells, it is important for most of these conditions to evaluate in living lungs as the complex structures and physiology and anatomy cannot be modelled in vitro.

Covid-19 is a global pandemic caused by SARS-Cov-2 virus, a coronavirus, that primarily manifests with respiratory symptoms. It can be transmitted by inhalation and enters the airway epithelium via the coronavirus envelope spike protein binding to the ACE-2 receptor. The route of uptake also involves protease activation. The genome has been sequenced and is available for research projects. While intense research efforts are in progress to identify a vaccine, concerns persist that an effective vaccine may be years away, and so alternative anti-viral therapies may be required and more effective in the shorter term. Nucleic acid therapeutics offer a wide range of therapeutic options and it will be important to evaluate these in animal models.

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

We hope to develop new therapies, such as gene therapy for respiratory diseases such as cystic fibrosis, primary ciliary dyskinesia and Covid-19, and learn how to deliver them most effectively and safely.

We aim to identify novel nanoparticle formulations that will be useful in the ongoing development of these therapies as well as methods of delivery by inhalation or systemic delivery.

We anticipate that numerous scientific papers, abstracts, posters and seminar presentations, will be produced from these studies that will directly benefit others working in this field.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the long term, our primary goal is to benefit the patients suffering from respiratory diseases such as CF, PCD and Covid-19 that have no other forms of treatment and face life-shortening problems along with the impact of these diseases on their everyday life.

In the shorter term, other scientists working in the same field will benefit in their research from our findings which we will share through the normal channels (publications, conference presentations, press etc.)

How will you maximise the outputs of your work?

Information on our findings and progress in this research will be disseminated to the scientific community by means of peer reviewed publications and oral and poster presentations at conferences and seminars. Where possible open access publications will be used as the vehicle for our research to maximise availability. Our websites will also be regularly updated to make information on our research freely available. Content will also be provided in a lay format. REDACTED will also publicise our findings and our institutional press offices will be notified of noteworthy findings for wider disclosure in the press. We will also communicate findings to companies at business/academic joint conferences and Knowledge Transfer Network meetings.



Species and numbers of animals expected to be used

- Mice: 5,000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Experiments, will involve anaesthesia followed by delivery of reagents such as nanoparticles or cells to the lung by different methods of inhalation, e.g. simply by breathing in an aerosol, by instillation via the oral or nasal route, or by injection into the blood through a vein. These procedure are all performed quickly taking less than 5 minutes per animal.

In some cases, where we aim to improve efficacy of nanoparticle delivery mice will be pre-treated with agents that improve access to some lung cells 2 hours before delivery of the test agents. In some other experiments mice may be pre-treated with alginates or other mucus modifying agents, particularly in Scnn1B mice, 2 hours before delivery of the test agents.

In experiments where we aim to use cells as the therapy, the lung may be pre-treated with a mild detergent, such as polidocanol, which removes cells lining the upper lung, to create space for the new cells to adhere and so repair the CF lung.

Mice will then be allowed to recover for 48 hours then treatment may undergo repeat dosing up to four times at intervals up to 7 days. Mice will be killed humanely by a schedule 1 method up to 1 month after the final dose, to assess efficiency of delivery, changes to gene expression, and safety of treatment by analysis of lung tissue sections or lung fluids.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The different treatments we are proposing generally have no observable effects. Occasionally there is weight loss at 24 h but by 48 h the mice are again putting on weight.

Administration of reagents into the lung by any of the proposed routes may cause a short period of hyperventilation from which mice usually recover within minutes.

We do not expect any effects on behaviour or tumour formation in the lung or anywhere else.

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

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



Wild type mice including reporter gene transgenic mice: - we have 20 year's experience of lung delivery of nanoparticles and other agents and rarely observe any side effects in these mice. 90% no effect; 5% mild; 5% moderate.

Scnn1b mice; from our experience over a period of about one year, we estimate that 20% of heterozygous mice die at the neonatal stage for unknown reasons but once weaned the mice show no signs of ill-health.

90% no effect; 5% mild; 5% moderate.

NOD/SCID mice are maintained in a sterile environment and will only be treated with sterile reagents or cells and so in our experience we do not expect to observe suffering of any severity. 90% no effect; 5% mild; 5% moderate.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 many diseases that affect the lung for which there are no effective treatments. These include inherited diseases such as cystic fibrosis. The aims of this research are to develop and evaluate new therapies for currently incurable diseases of the lung. Mice are the smallest animals with lungs that it is practical to use in laboratory experiments. Structurally the lung is a complex branching structure with many cell types that vary at different stages of the branched structure. In the upper parts of the airways, the trachea, bronchi and bronchioles are lined with cells that produce numerous hair-like projections called cilia, that wave to and fro, moving mucus and embedded particles up towards the mouth in a process called mucociliary clearance. These anatomical and physiological features of the lung cannot be replicated in vitro. Access to the different stages of the lung by methods and routes of delivery also cannot be investigated in vitro.

What was your strategy for searching for non-animal alternatives?

We will use cultured cells for many experiments including air-liquid interface cultures where human primary epithelial cells differentiate to form the epithelial cell types including mucus-producing and ciliated cells. These cells also express ion channels including the chloride and sodium channels involved in cystic fibrosis. We will do as much as we can in these models to develop the most promising therapies.

Why were they not suitable?

The lung is a complex organ and there is no way to reproduce that complexity in a dish in the lab. Cells are cultured on a flat dish where the lung is a complex 3-dimensional organ, so live mice are necessary for these studies to have maximum scientific value.

Reduction



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

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

Animals will be used from breeding Protocols 1 and 2 while other wild type mice will be purchased from authorised suppliers. We already have a lot of experience in these kinds of experiments. Power calculations will be used to predict numbers of mice required to obtain statistically valid data with the minimum number of mice. These calculations are based on more than 20 years' experience of in vivo lung delivery and analysis of gene expression and so these power calculations will usually be based on established experience. In some cases, where that prior experience is not available, we will perform pilot studies to generate data for this purpose.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Local statisticians will be available to advise on experimental design and we will use the NC3Rs Experimental Design Assistant.

As part of this project we will continue to develop and apply non-invasive imaging modalities including bioluminescence and fluorescence imaging to monitor and assess tissue responses, in the hope that this will allow the identification of earlier endpoints. The use of live imaging may also lead to a reduction in animal numbers.

What other measures apart from good experimental design will you use to minimise numbers?

Pilot studies will be performed for each protocol step as required to determine the minimal number of mice to generate valid scientific data. We also have more than 10 years of experience already in performing such experiments and so that experience will be valuable in our design. Imaging experiments in real time with the same animal will help to reduce numbers of individual animal measurements.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We intend to use mice, since a major aim of this project is to develop treatments for inherited genetic disease and there are many mouse models of human genetic disease. They have been designed to carry defects in the same respective genes, and in some circumstances show very similar lung diseases. Importantly, exactly the same gene



therapy vector or cells that we use to treat a mouse can then be used to treat a human being.

Some of the mice have genetic defects that cause thickened mucus in the lungs, as found in CF, and so provide a more refined, informative model than wild type mice.

The reporter mice we propose to use are specifically informative on efficiency of delivery for example of CRISPR or mRNA therapies.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We require animals that have lungs and the mouse is the smallest and most practical option. Adult mice will be used mostly as they are easier to work with in terms of size and better represent the lungs of patients. The developing lung for example in neonates may not contain the fully differentiated epithelium that we aim to treat and correct.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The animal models of disease to be used in this programme may not always mimic the human condition precisely but have been chosen because they are recognised by those in the field to be the most appropriate available. We have extensive experience in the development and refinement of these models, over a number of years allowing us to use the smallest species possible, reduce numbers of animals, limit invasive procedures and limit discomfort to a minimum consistent with a reproducible, statistically significant pathologic outcome.

The Named Veterinary Surgeon will be consulted whenever necessary. When work under terminal anaesthesia is involved, the level of anaesthesia will be maintained at sufficient depth for the animal to feel no pain.

We will continue to monitor our own practices and the literature, and will incorporate new approaches whenever possible to further refine, reduce and replace the use of animals. For example, we are investigating inhaled aerosol delivery methods with a small mask for the mouse rather than the more invasive instillation methods.

In general, our procedures and analytical protocols have been refined to allow us to use mice in most of our studies. Mice are also the species of choice due to the greater availability of genetically modified strains.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow ARRIVE and PREPARE guidelines for planning experiments as well as the NC3Rs experimental design assitant for experimental design and for administration of substances by different routes.

How will you ensure you continue to use the most refined methods during the lifetime of this project?



I am a registered user of the NC3Rs newsletter and will regularly check the NC3Rs website for news of advances. We will also communicate with our local qualified staff for recommendations and updates on advances in 3Rs and how best to implement them.

Explain the choice of species and the related life stages

These experiments to develop novel genetic and cell-based therapies for incurable genetic respiratory diseases such as cystic fibrosis and primary ciliary dyskinesia. Mice are the smallest laboratory animal that have lungs similar in anatomy and physiology to those of humans. We aim to use mice to confirm delivery of our novel therapeutics to the lung, an organ which contains many different cell types and a complex, branching structure that cannot be mimicked in the laboratory. Live animals also produce physiological responses to treatments that cannot be mimicked any other way which may be important for assessing efficacy, safety and toxicity of treatments. Adult mice will be used in most experiments although we may also use neonatal/pre-weaning Scnn1B mice to assess enhanced survival by treatment early in life.





38. Developing pre-clinical models of Prader-Willi syndrome

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

Development and characterisation of preclinical mouse models which faithfully recapitulate clinical aspects of Prader Willi Syndrome (PWS). Assess the use of these PWS REDACTED as pre-clinical models to further understand, validate, test therapeutics and potentially treat symptoms of PWS.

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

Why is it important to undertake this work?

PWS is a genetic neurodevelopmental disorder that occurs approximately once or twice in every 30,000 births. It is known to be caused by the absence of a set of paternally expressed genes on human chromosome 15. Symptoms are multiple and debilitating,



including hypotonia and feeding problems in infancy, intellect and learning disabilities, anxiety, behavioural and sleep abnormalities, incomplete sexual development, skin picking and severe hyperphagia leading to obesity. Patients also have a higher tolerance to pain which can lead to injury and infection. Later in life patients can develop psychosis and depression as well as secondary metabolic problems linked to obesity which are life limiting. Additionally there is an increased risk of sudden unexpected death at any age, with a prevalence of about 3%.

Although the group of genes involved in PWS are known, it is not understood which of these genes contribute to each symptom, and the mechanisms through which the symptoms are caused.

The only approved therapy for PWS is growth hormone, which helps with some symptoms but has no impact on hyperphagia, anxiety, sleep or behavioural and intellectual challenges. Symptoms of obesity can be helped with a strictly controlled diet, however the drive to eat is always present and exacerbates anxiety, depression and behavioural problems as children that feel hungry are denied food from their carers.

Previous models of PWS have been of some use but have been limited by sub-optimal model development using older transgenic technologies, incomplete phenotypic characterization, the heterogeneity of assessment methods and the lack of comprehensive, rigorous and standardized phenotyping. This programme of work aims to address these issues, firstly to generate meticulously quality controlled genetically altered REDACTED carrying modifications in PWS genes (this step is carried out on another licence), secondly to phenotype these lines through multiple phenotyping pipelines resulting in comprehensive data regarding all aspects of PWS that can be recapitulated in mouse models. Finally, validated models along with any mechanistic information that has been garnered from their study, will be used for basic research to better understand the pathophysiology of PWS and to trial new therapeutic drugs to treat humans with PWS.

A more complete characterisation and validation of mouse models of PWS may open up new avenues for understanding of the genes, proteins and mechanisms involved in this disorder and discovery of drug targets and potential therapeutic approaches. To ensure this characterisation and validation is done in the best possible way, a collaboration has been set up involving specialist researchers and clinicians throughout Europe and the United States who regularly meet to discuss and review plans and progress.

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

We believe that development and validation of preclinical mouse models of PWS can be best accomplished through a collaborative effort that will leverage the know-how and expertise of various laboratories beyond what could be achieved by laboratories working in isolation. In addition, the network will provide comprehensive and unbiased data across developmental stages and phenotypic abnormalities as well as recommendations and best practices on mouse models of PWS for the scientific community. This will help guide researchers to select the most relevant mouse model and protocol for their study. Because PWS is a complex disease affecting many functions that may be inter-related, comprehensive phenotyping across multiple systems may provide important information on the relationship between these different functions (e.g sleep and cognition). In addition, the network will serve as a preclinical drug screening platform that will help accelerate the therapeutic development for PWS.

There will be four main outputs from this project.



Comprehensively evaluate at least five mouse models of PWS and make available to the scientific community.

Complete datasets on all PWS models available to all researchers to analyse and interrogate.

Advice on best practice to researchers using PWS models.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the initial stages the outputs will benefit the wider scientific community as data generated from this project will inform future studies.

However, there are very few treatments for PWS as the mechanisms are so poorly understood. It is reasonable to think that once further mechanisms and targets have been highlighted through these studies, there may already be drugs available that could alleviate some of the PWS symptoms. Efficacy testing these drugs in validated preclinical models will be the first step in getting new treatments to PWS patients. The aim of this licence is to validate and characterise these models and make them available for efficacy testing to other researchers on other project licences.

How will you maximise the outputs of your work?

The project is led by FPWR, a patient research-driven organization who is committed to improve and validate preclinical mouse models of PWS for the benefit of all. FPWR is regularly communicating about the importance of the project for the scientific community including pharmaceutical industries developing therapies for PWS, as well as to patients and caregivers. Data and recommendations will be shared with the community that will include access to data, procedures and publication in a scientific peer-reviewed journals. These recommendations will be enforced through the FPWR grant program. In addition, mouse models will be made publicly available through the EMMA (European Mouse Mutant Archive) network.

One of the critical remits of this project is to increase the quality and usefulness of mouse models for PWS. This includes informing the community on which symptoms are not recapitulated and which models are not appropriate, as well as models and symptoms which are.

Species and numbers of animals expected to be used

- Mice: 41500

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



Many of the mice on this licence will be used only for breeding. This is due to the complex nature of PWS and the need to carry out several steps of breeding to get to a cohort that can be studied. We will also maintain breeding lines at Harwell to ship to collaborators for use on other projects authorised to accept GA lines.

In terms of testing at MRC Harwell, approximately 3500 mice will go through one of three pipelines. A pipeline that focuses on behaviour aspects of PWS, a second pipeline that focuses on metabolism and a third on nociception. Each pipeline involves 12-15 tests, most of which are non-invasive, a small number (two in each pipeline) involved anaesthesia used for immobilisation only. Mice are then finally anaesthetised and a terminal bleed carried out. All pipelines are expected to end by 30 weeks of age.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Humans with PWS suffer from hypotonia, feeding problems and failure to thrive, insatiable hunger drive, excessive eating (hyperphagia) leading to life threatening obesity, hypogonadism, cognitive impairment, sleep impairment, behavioural features which commonly include obsessive compulsive behaviours, anxiety, temper tantrums, and mental illness with a high prevalence of autism, psychotic episodes, and affective disorders. It may be that some or all of these are recapitulated in various different GA lines on this licence. Previous models of PWS have shown only a subset of these clinical signs, it is also not clear how some of these signs will manifest in mouse models, for example psychotic episodes and temper tantrums. However, it should be assumed that it is possible that a mouse could display all of these symptoms and potentially other unknown phenotypes that are not seen in humans.

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

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

7500 mice on the mild breeding protocol are not expected to suffer any adverse effects and the vast majority will not reach higher than a sub-threshold severity.

On the moderate protocol, it should be anticipated that any mice carrying the disease causing phenotype could exhibit a moderate phenotype. Other genotypes will also be born from these crosses, so approximately 50% of the 7500 mice may suffer a moderate severity.

3500 mice on the metabolic and behavioural phenotyping protocol are all expected to reach a moderate severity. This is partly due to the phenotype of the mice, in which the genetic alteration could lead to a moderate severity in around 50% of the mice (the other 50% being unaffected controls). However, all mice will reach a moderate severity due to a small set of the phenotyping tests causing moderate suffering, for example overnight fasting for blood sampling, or fear conditioning. Whilst these moderate affects will be short lasting they will increase the maximum severity of all animals on this protocol to moderate.

Similarly, of the 600 mice on the nociception pipeline, around 50% may reach a moderate severity due to the nature of the genetic alteration. Control mice should suffer no more



than mild severity as the phenotyping tests included should result in no more than mild, transient discomfort.

What will happen to the animals at the end of the study?

- 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?

Prader-Willi Syndrome (PWS) is an imprinting gene disorder caused by loss of expression of a cluster of exclusively paternally expressed genes on human chromosome 15. The PWS imprinted gene cluster includes six paternally expressed genes and two major clusters of the paternally expressed small nucleolar RNAs in humans. Non-mammal vertebrates lack at least five of these genes. In mice the PWS imprinted gene cluster is found clustered together in a very similar way to the human gene arrangement, with the exception of one primate specific gene.

PWS is a complex disorder involving multiple genes and affecting tissues throughout the whole body, additionally the mechanisms through which the symptoms occur are poorly understood. Only by studying a whole organism will we be able to disentangle which genes are responsible for which symptoms and under which circumstances. There is not yet enough knowledge to study these changes in tissue culture as it is not clear which tissues/cells should be utilised.

The complex characteristics of PWS together with the well conserved genetics between the human and the murine region lend importance to the development of mouse models for PWS. Because of the changes in the disease manifestations across lifespan, mice will be analysed at three developmental stages (neonatal, juvenile and adult), across a comprehensive battery of tests to assess multiple system function including motor system, metabolism, cognition, social behaviours, sleep, circadian rhythms, temperature regulation, respiratory function, gastrointestinal and other functions that are impaired in individuals with PWS.

What was your strategy for searching for non-animal alternatives?

Some cell models for PWS have been developed. They comprise of fibroblasts, dental pulp cells and induced pluripotent cells derived from individuals with PWS as well as cultured cells originating from mouse models of PWS. Studies have shown defective PWS gene receptor trafficking, neuronal developmental and signaling defects. However, these cell models have not helped to discover molecular and cellular signatures of the disease and the extrapolation of the cell culture phenotypes to human symptoms in such a complex disorder cannot be done without further information, which needs to be generated in an animal model.

FPWR is also funding efforts to develop alternatives to animal models and has recently launched a PWS iPSCs biobank (<https://www.fpwr.org/ipsc-biobank>) to foster the development and characterization of cell models. As with the mouse models, FPWR are committed to building a repository of highly quality controlled and well characterised iPSCs



which can be used by researchers around the globe. Developments in stem cell research will be continually assessed and any possibility to move research to stem cells or organoids will be investigated as it become available.

Why were they not suitable?

The biology of PWS is poorly understood, whilst the genes in the affected region have been identified it is not clear which of the genes causes the specific phenotypes or whether some genes are working in combination. PWS is a multi-system disorder comprising aspects of behaviour, sensory and metabolism. Since there are multiple genes acting on multiple organs it is difficult to model this in an organoid system. By using mouse models to increase understanding of which genes effect which tissues, and how they influence each other, it may be possible in future to build sufficiently complex tissue culture conditions. At present there is not sufficient knowledge to do this.

Reduction

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

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

Numbers of animals used for phenotyping is based on power calculations using previous baseline data. This takes into account both the effect size and variability of data for each test and also the pipeline of tests as a whole. For example, previous baseline data for C57BL/6J males, shows that for open field total distance moved, the average is 2108cm, with a standard deviation of 510. To pick up a 25% drop in activity by the GA lines, with a power of 0.8 and a type I error rate of 5%, we would need $n=15$

Current estimates are that we will need a sample size of 15 per sex and genotype for the behaviour and metabolic pipelines, and a sample size of 10 for the neonate and nociception pipeline. However this will be reviewed as baseline data is collected for more of the tests.

Additional cohorts sent to collaborators will be larger as in-depth behavioural testing requires larger sample sizes of 20-25 depending on the paradigm.

Pipelines have been designed to gather the most meaningful data. Tests which can inform each other will be carried out on the same mouse to remove inter animal variability and increase the power, thereby decreasing the sample size.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

A local bioinformatician has been involved in helping us calculate sample sizes based on the pipeline and taking into account previous data. Online power equation calculators have been used to look at individual tests.

SOP's have been written and used routinely for previous projects. This standardises the way the data is collected and reduces the variability and therefore the sample size.



What other measures apart from good experimental design will you use to minimise numbers?

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

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

All animals will be randomly assigned to cages and technicians and data analysts will be blinded to genotype until the study is completely finished and data analysis complete.

The aim of this project is to produce well defined clinical ready models to be used by the entire PWS research community. As such, large biobanks of tissues will be set up and tissue collected from all models on termination of the experiment. Once the data is validated and phenotypes assessed, tissues and data will be made available to the community.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

It is not yet clear which GA strains will best model PWS. In this study we will characterise mice with genetic changes in the PWS region. Initially these will be global deletions, however depending on the phenotype of these mice we may move to conditionally deleted mice, removing the gene in a tissue or time specific manner rather than throughout the whole organism for life. A full characterisation will be carried out to assess any overlap of symptoms between humans and mice. It is possible that some of these GA strains will not characterise the disease well. We will publish both the positive and negative data on each strain. It is hoped that this research will provide data for the scientific community, full characterisation will inform researchers which models they should use to study certain aspects of PWS, and which models are not useful.

Mice are generated and bred on a co-isogenic inbred lines to remove any confounding factors of genetic background.

Test	Why is this the most refined method?
Ear biopsy	Mice need to be ear clipped for identification and the same piece of tissues is used for genotyping. Genotyping



	<p>protocols have been optimised to use this very small samples for all types of genotyping currently carried out at MRC Harwell. This is more refined than other methods such as tail biopsy.</p>
Induction of transgene expression	<p>In most cases this will be done by oral gavage on five consecutive days. Previous data has shown that this number of doses is necessary for sufficient induction of expression. This is more refined than injection, as whilst it involves restraint there should be no pain. Injection may be necessary depending on the type of recombinase line used and the time at which it needs to be activated.</p>
SHIRPA	<p>SHIRPA is an observational test that involves placing the mouse in an arena or a jar and looking for abnormalities. This is non-invasive and can provide a lot of phenotypic information that can be followed up in further, more complex tests. This is also a good opportunity to pick up any more subtle welfare concerns.</p>
Open field	<p>Open field gives us data on the reaction of the mouse to a novel environment. This arena is anxiety inducing in that the mouse has not seen it before and the light is relatively bright. There are no smells or sounds that should cause any further stress to the animal.</p>
Grip strength	<p>The grip strength tests is the quickest, least invasive way of measuring animals muscle strength. The test lasts for less than one minute and should cause no pain, only stress induced by handling.</p>
Rotarod	<p>Rotarod is useful to measure activity when the mouse is encouraged to run by placement on a moving rod. It is also a useful cross laboratory comparison to ensure reproducible phenotypes. Where possible, we will use voluntary wheel running to assess co-ordination or motor function as there is less stress induced</p>



	from handling and the animal can choose whether to use the wheel or not.
Gait analysis	This involves placing the mouse in an arena and videoing for a short time. This is the most refined method of running this test as, unlike some other gait analysis equipment, it does not involve any stimulation to force the mouse to move.
Acoustic startle and pre-pulse inhibition	For this test it is necessary to assess the response of the mouse to an audible stimulus. Whilst the stimulus itself is anxiety inducing, efforts are made to reduce stress by having a constant background noise and carrying out the test in the dark. A protocol has been developed over many years to give useful data over as short a time as possible.
Spontaneous alternation	This is none invasive and involves placing a mouse in an arena and allowing it to explore. Light levels are set so them mouse can see but are not intended to by anxiety inducing.
Elevated plus/zero	This is non-invasive and involves placing a mouse in an arena and allowing it to explore. Light levels are anxiety inducing in some areas of the arena but other areas are darker. This is necessary as the test is assessing the preference of the mouse to the darker or lighter areas.
Social recognition	This is non-invasive and involves placing a mouse in an arena and allowing it to explore. Light levels are low to reduce anxiety. In this test another mouse is present in a section of the arena. This mouse is in a small cage to stop any aggression but the scent and sight of it is needed to assess behaviour of the test mouse to a social stimulus.
Novel object recognition	This test is now carried out in a home cage environment. This reduces the stress on the animal and the novel object may be seen as a form as enrichment.



Temperature taking	This is done using a rectal probe that should cause no more than transient discomfort. The animal is only lightly restrained and the test is done in the shortest time possible, usually less than one minute.
Urine collection	Mice are held and urine caught in a container, or in some cases mice are placed on a plate and allowed to explore while urine collects below. In both cases this is less stressful than placing the mouse in a metabolic cage and collecting urine overnight.
Light dark box	This is non-invasive and involves placing a mouse in an arena and allowing it to explore. Light levels are anxiety inducing in some areas of the arena but not others. This is necessary as the test is assessing the preference of the mouse to the darker or lighter areas.
Ophthalmoscope	For this test the mouse is held and the eyes examined. This is more refined than more in depth imaging as it does not involve general anaesthetic. However, the data collected is more subjective and there may be times when in-depth imaging is needed, in which case mice will undergo optical coherence tomography (imaging of the eye under general anaesthesia).
ECHO-MRI	This test involves less than one minute of light restraint whilst body composition is measured. This is more refined than alternative tests which require general anaesthesia and a longer time to gather the data.
Blood sampling	Blood samples are collected to a maximum of 15% total blood volume of the animal, which has shown no adverse effects in previous studies. Samples are taken from the tail vein using a very small cut and mice have local anaesthetic applied to the area twenty minutes before the bleed takes place.



Tolerance tests	<p>Blood samples are collected to a maximum of 15% total blood volume. Fasting for this tests is usually 18 hours due to the need to allow animals to use up glucose supplies and enter gluconeogenesis. However, where possible fasting times will be reduced (e.g. 6 hours maximum for insulin tolerance).</p> <p>This test is more refined than other methods of measuring glucose which require surgery to implant indwelling catheters.</p>
Video Tracking	<p>This is non-invasive and measured in the home cage, however due to recording sleep equipment animals have to be singly housed.</p>
Circadian	<p>This is non-invasive and measured in the home cage, however due to recording equipment animals have to be singly housed.</p>
Voluntary wheel running	<p>This is non-invasive and measured in the home cage, however due to recording equipment animals have to be singly housed. This is a refinement on the rotarod test as animals can chose whether to run on the wheels.</p>
Standard calorimetry	<p>This is non-invasive in home cage like equipment. Bedding is provided and animal shelters are used when activity measurements are not needed. This test is used to measure oxygen and carbon dioxide levels and use them to calculate metabolic paratmeters.</p>
Advanced calorimetry	<p>This is non-invasive in home cage like equipment, however the floor is gridded and no shelters or bedding are available. As this is more stressful than standard calorimetry it will only be used when it is essential to have parameters that can only be measured with this equipment. This test is used to measure oxygen and carbon dioxide levels and use them to calculate metabolic parameters. In this equipment it is also possible to alter the temperature</p>



	and lighting regimes, as well as measure body weight and food weight.
Metabolic cages	Mice are housed for as short a time as possible, usually 20 hours. This length is needed to collect a large amount of urine and assess overnight food and water consumption. Red houses are placed in the cages to provide shelter and respite from the grid floor.
Home cage monitoring	This is non-invasive and measured in the home cage. After initial insertion of a microchip this test involves no further pain, suffering or distress to the mouse. This generates large amounts of data with no adverse welfare effects. The main parameter measured is activity but videos may be used to assess other behaviours.
Stress induced hyperthermia	This test is necessary to assess stress without relying on activity. Mice will be closely monitored throughout and protocols have been designed for the shortest time possible. At all times mice are in a home cage but will be singly housed and with limited bedding as it is necessary for this to be a stress inducing environment.
Cold test	Mice are placed in a cooled environment (approximately 4 degrees centigrade) for up to 6 hours. Mice are closely monitored and removed if the temperature drops too low. This test is used to assess how well the mouse can regulate it's own body temperature.
Hot plate	This test has been refined so the mice are removed from the apparatus at the first sign of a response to the heat. Two technicians and multiple mirrors are utilised to ensure the first response is not missed. This test assesses the thermal pain response of the mouse.
Von Frey	This involves putting pressure on the mouse paw with a filament. The number of filament presentations has been reduced



	in this method as legacy data on miceshows the need to focus on a particular size of filaments. Mice are habituated to the arenas to reduce stress. This test assesses the mechanical pain response of the mouse.
DEXA/X-ray	DEXA and X-ray are not done using one piece of equipment which has been refined to allow constant observation and the use of gases anaesthetic. Both tests can be done together to reduce the need for further anaesthesia. This test measures bone mineral content and density, as well as producing an image to assess bone structure and abnormalities.
Echo-ultrasound	These tests are done under gaseous anaesthesia. When mice require both tests they are carried out under one anaesthetic. Potential skin damage from shaving has been mitigated by the use of hair removal cream. These tests image the heart to Echocardiogram measure structural abnormalities and produce a reading of the electrical activity of the heart.
Auditory brainstem response	This test is done under general anaesthetic due to the extremely sensitive nature of the measuring electrodes and the need for a completely sealed sound proof box. Mice are monitored at all times through a window and the electrodes themselves should cause no pain. This test measures the electrical response of the brain when specific sounds are presented.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Prader-Willie Syndrome (PWS) is caused by 6 genes on chromosome 15 not being expressed and therefore not making any proteins. In this same region are sections of DNA that code for another type of nucleic acid, RNA, these RNA's control several processes within the cell and are also missing in PWS patients. The DNA that codes for these genes and RNA are found together at the end of chromosome 15 in humans and are all lost together by large deletions in PWS. In mice, with the exception of one gene which is



specific to primates, this cluster of genes is also found together on chromosome 7. Therefore it is possible to create the same type of deletions in mice as it is in humans. Non-mammalian vertebrates lack 3 of these genes and no similar sequences to another two genes of these genes were found in chicken and fish. Making these animals less useful to model this particular disease.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

For all tests it is important that the animal has no additional stress, therefore mice are handled calmly and habituated to testing rooms as well as arenas if possible.

For all tests mice are only housed in modified cages or arenas for the minimum time needed to gather meaningful data. Mice undergoing phenotyping tests have increased monitoring and are removed from tests if they appear to be suffering from an adverse stress reaction, or other unexpected adverse effects of the phenotyping tests. Mice which have had anaesthesia have extra monitoring until fully recovered and extra checks when back in the holding rooms. When general anaesthetics are necessary, the combinations with least adverse effects will be used, for example for all tests inhalation anaesthetics will be used, with the exceptions of Auditory Brainstem Response and Optical Coherence Tomography which cannot be carried out with the mouse on a face mask. Pain from tail bleeds is reduced by using local anaesthesia.

This licence involves no surgery.

Pipelines are designed with thought 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 be followed to ensure experiments are conducted in most refined way?

Routes and volumes for administration of substances are taken from LASA guidelines.

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

Standard operation procedures for most tests have been generated using data and expertise from multiple animal houses and can be found at <https://www.mousephenotype.org/impress>

ARRIVE guidelines will be followed at all times.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Projects leads will attend general 3R's 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 research team will attend PWS specific conferences that focus on all aspects of the disease, from humans, to model organisms to *in-vitro* and *in-silico* work. Any new developments which could impact these studies will be discussed with the Pre-Clinical Models Network, a panel of experts set up to design and oversee this project.

Explain the choice of species and the related life stages

It is necessary to use mice for this study due to the similarity of their genome to the human genome in the PWS region. PWS is characterised by the loss of paternally expressed genes in a section of human chromosome 15. Whilst some of the genes in this region are found in non-mammalian vertebrates, at least three of the genes are not found. We need to study the region as a whole and the interactions between the genes, this therefore cannot be done in species other than mammals. Mice contain almost the full complement of PWS genes on mouse chromosome 7, with the exception of one gene which is primate specific.

We are studying neonatal, juvenile and adult mice due to the nature of the disease in humans. Symptoms are seen early in life in newborn babies and these symptoms change overtime until adulthood. To study these different characteristics it is necessary to study the mice at various life stages, from birth, through to adulthood and ageing. Where GA mice are not viable we may study embryos to help us understand why the mice cannot survive without a particular gene or set of genes.





39. Development and function of immune cells in the uterus

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

The aim of this project is to determine how immune cells in the uterus develop and how they contribute to the success of pregnancy.

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

Why is it important to undertake this work?

Immune cells in the lining of the uterus help the placenta to implant at the beginning of pregnancy, and are involved in the initiation of labour at the end. Both of these processes have to be carefully regulated in order for pregnancy to succeed. For example, insufficient placental implantation can lead to miscarriages and disorders of pregnancy such as pre-eclampsia. Similarly, going into labour too early or too late are both associated with a greater likelihood that the baby will die.

My lab uses samples from human pregnancies to look at how these immune cells function in health and disease, but there are limits to what we can do using these approaches. Therefore, we will support and inform our human work by doing studies in mice, which



allow us to specifically remove and add back immune cells to see how they develop and how their presence or absence affects pregnancy. These experiments will help us to identify cells and pathways that we can target in the future to improve outcomes of pregnancy for mothers and babies.

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

Advancement of scientific knowledge

The major output from this project will be new information about how immune cells develop in the uterus, and how these immune cells can help pregnancy to succeed. I will disseminate this information through scientific publications and presentations, as well as to my clinical colleagues and to the public through my engagement work with school pupils and patients.

Identification of targets that can improve outcomes of pregnancy for mothers and babies

A secondary output relates to the possibility that laboratory finding could be translated into a clinical setting. By integrating the animal work outlined here with the work that I do with clinical samples, and by working closely with my clinical colleagues, we may be able to identify new cells and pathways that could be targeted to improve the outcomes of pregnancy for mothers and babies, for example by reducing the likelihood of disorders of pregnancy such as recurrent implantation failure, miscarriage, pre-eclampsia and preterm birth.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Scientists

In the short and medium terms, the main beneficiaries of this project will be other scientists studying pregnancy, who will be able to use its findings to inform their own work.

Clinicians and patients

In the longer term, potential beneficiaries include couples who are struggling to conceive, and those who experience recurrent miscarriages and disorders of pregnancy such as pre-eclampsia and preterm birth. This study, and later ones informed by it, may help with the design of diagnostic tools and clinical interventions that can improve the outcomes for these families.

How will you maximise the outputs of your work?

I will maximise the outputs of this work within the scientific community by publication of my findings in peer reviewed journals and presentation of my work at national and international meetings. I will also publish negative findings, to avoid unnecessary duplication of work.

In the course of my group's work on human tissues, we collaborate closely with clinicians working in the field of reproduction, and will communicate the findings to our clinical colleagues, in order to facilitate any potential translation of our findings.



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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

About 75% of the mice used in this project will be genetically modified animals used for breeding. Males and females will be kept together and allowed to have pups until they are 15 months old. At the end of the breeding period, the mice will be killed humanely.

About 20% of the mice used in this project will undergo minor procedures, such as receiving an injection or giving a blood sample. This will help us to characterise their immune systems in more detail.

Less than 5% of the mice in this project will undergo irradiation to "make room" for new immune cells, which we will inject into the mice, to grow. The mice will be kept for between eight and sixteen weeks to allow their new immune system to "settle" before we use them in further experiments.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The genetically modified animals that we will use in this project may have defects in their immune systems, but when kept in the very clean conditions that we use in our animal facility, their lives will not be very different from those of normal mice.

The mice that undergo minor procedures, such as receiving an injection or giving a blood sample, will only experience momentary pain or discomfort.

Mice undergoing irradiation prior to transfer of immune cells may experience more severe adverse effects, similar to those that a person having radiotherapy as a treatment for cancer. They may have gut problems, such as diarrhoea, and are more likely to get an infection. When this happens, it usually occurs within the first two weeks after irradiation. The mice will be more carefully monitored during this period and any mice that lose a lot of weight or behave as if they are ill 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 species)?

Mild: 95% of animals

Moderate: 5% of animals



What will happen to the animals at the end of the study?

- 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, I will also examine immune cells taken from human tissues and this will reduce the number of mice I have to use. However, access to human tissue is limited and there are also certain kinds of studies that cannot be carried out on humans. Working with mice allows me to make changes to the animals – such as genetic modification – that would not be ethical or practical in humans. Therefore, using the mice will provide valuable additional information that could not have been collected from work with human samples alone.

What was your strategy for searching for non-animal alternatives?

This project also involves the use of primary human tissues and cells, and cell culture approaches.

Why were they not suitable?

These approaches are useful and will provide important information, but there is a limit to what they can tell us. In particular, using mice makes it possible to examine the complex interplay between immune cells (which may move around the body) and cells in the uterus, such as those of the placenta and blood vessels. It also gives us the ability to determine the effects of particular genes by using knockout animals. Neither of these is possible using patient samples or cells in culture.

Reduction

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

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

I have estimated the number of genetically altered animals that I will breed based on a rate of 300 animals/year for the duration of the project. This is roughly in line with what I currently breed, allowing for animals bred for tissues only and those animals produced as a by product of breeding mice of interest, which do not have a useful genotype.

For experimental protocols, where suitable preliminary data exists in the literature or in my laboratory, I have used it to calculate the required experimental group sizes. Where such data does not exist, I will first carry out small pilot experiments to determine the required group sizes.



What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

I have designed efficient breeding strategies using guidance from JAX laboratories, especially for conditional knockouts, found here:

<https://www.jax.org/news-and-insights/jax-blog/2011/september/cre-lox-breeding-for-dummies>

I have designed the experiments using statistical approaches that I learnt in the mandatory statistical training that I attended in order to be allowed to hold a project licence, and summarised here:

<http://www.3rs-reduction.co.uk/>

What other measures apart from good experimental design will you use to minimise numbers?

I will reduce the number of animals used by:

- Efficient breeding strategies
- Statistical approaches to determine the smallest sample size that can detect an effect
- Taking multiple tissues from each mouse where possible
- Taking multiple measurements from each mouse where possible
- Tissue sharing with other groups 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The vast majority of mice in this project will suffer no more than transient pain or discomfort, with no lasting effects. Those mice that undergo irradiation before immune cells are transferred to them may experience gut trouble and increased susceptibility to infection, but this is an unavoidable consequence of the need to use radiation in these experiments.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The two groups of animals with similar reproductive immunology to that of humans are non-human primates (for example, macaque monkeys) and rodents (for example, mice). I have chosen to use mice in this study because they can be considered less sentient than



non-human primates and because a number of genetically modified strains of mouse are already available. The fact that these strains have already been made will mean that we do not have to start from scratch, which would require many more mice.

In this study, which requires animals to mate, they must necessarily be conscious adults.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The following refinements are described in the protocols:

- Increased monitoring following irradiation
- Avoiding the unnecessary use of antibiotics
- Increased monitoring of pregnant animals following intraperitoneal injections
- Administering topical analgesia to avoid pain during repeated blood sampling

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

I will use guidance published and kept up to date by NC3Rs:
<https://www.nc3rs.org.uk/3rs-resources>

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I receive monthly email updates about 3Rs issues from NC3Rs. Our animal facility is also very good at communicating new ideas about 3Rs through email updates, posters displayed in the facility and through conversations with staff when my team and I are working in the facility.

Explain the choice of species and the related life stages

I have chosen to use mice because they have a similar immune and reproductive system to humans, and because genetically altered animals that allow me to define the roles of different immune cells are available. Because the project looks at the immune system in pregnancy, I have to use adult, pregnant animals.





40. Development of therapies against degenerative diseases

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The aim of this programme of work is to use animal models to assist in the development of new therapies for the treatment of degenerative diseases (such as Parkinson's, Huntington's disease or amyotrophic lateral sclerosis).

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

What are the potential benefits that will derive from this project?

The primary benefit of this study is the identification of best drug candidates suitable to treat the previously intractable degenerative diseases in human. The molecules selected in these studies will be further developed in the clinic.

Due to the increased prevalence of neurodegenerative diseases and the lack of effective treatments available the impact of this project might be very high.

Species and numbers of animals expected to be used



What types and approximate numbers of animals will you use over the course of this project?

Mice are required as they provide good models of human diseases. We expect that ~ 20 000 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.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Mice will be maintained in a social environment and environmental enrichments will be provided to improve their wellbeing. Both genetic (GM) and pharmacological models of degenerative diseases will be used. Mice whose disease progression will be monitored closely, will be kept until they develop the disease symptoms but will be culled at the latest when the clinical signs like weight loss or hindlimb paralysis are present. Some mice will be treated with pharmacological substances. The administration might cause intermittent distress but no lasting adverse effects are expected. In most cases substances will be administered by the oral route. Behavioural testing will be used to assess the disease phenotype. In most cases behavioural tests that will be applied to mice are non-invasive and are not expected to cause any lasting distress or harm. On very rare occasions mice might be subjected to a more invasive test, which involves touching a plate of increased temperature (max. 55°C), however, a mouse is free to remove its paw or tail once it starts to feel uncomfortable. 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 and pathology as well as drug efficacy will be monitored by postmortem tissue analysis. In one of the genetic mouse models to be used, the genetic modification leads to limited number (<5%) of sudden deaths not preceded by detectable signs of disease, and so not possible to predict. We will search for refinements to our methods, protocols and monitoring procedures in order to decrease the incidence of mice found dead suddenly and unexpectedly.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

We have a group of molecules with a high potential to ameliorate degenerative diseases. These molecules are being extensively studied in order to select the best drug candidates to be moved forward to clinical trials.

Testing in animal models is an essential prerequisite of clinical trials. Prior to being used in humans, there is a legal requirement for virtually all new disease treatments to be tested in animal models of the disease(s) in question. The mouse is best suited for this work since, of all existing models, mouse models are highly relevant to the human diseases.

Whilst tissue cultures provide some useful information, they cannot fully replace mouse models. They do not provide the physiological conditions, the complex interactions



amongst different cells and the metabolic treatment of a drug that is seen in a normal living body.

Before being tested in mice the candidate drugs will be carefully selected by tests on cell lines or in ex vivo cultures and only the most promising candidates will be tested in vivo.

The work in mice that we propose is essential to validate our approaches and may have a big impact on human health.

Reduction

Explain how you will assure the use of minimum numbers of animals.

The minimum number of mice will be used in each experiment, while ensuring that the numbers are large enough to obtain solid and reliable data. The mouse numbers will be chosen based on published data, previous studies or determined based on statistical power analysis.

To reduce the sources of variability and bias mice will be randomly assigned to experimental groups. Experiments will be run in a blind fashion.

Mouse breeding will be carefully monitored to ensure that surplus animals are not generated.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The most refined and the most appropriate models to answer the question asked will be chosen for the studies. Mouse models of common degenerative diseases exhibit the essential features of the human diseases. It is necessary for the validation of disease modifiers identified in cells to address their relevance in mouse models. At present, no valid alternative to animal models exists.

Mice will be housed in a social environment with environmental enrichments in order to improve the wellbeing. Only mice needed for the scientific purpose will be kept up to the moderate severity limit. Signs of the disease and the adverse effects will be limited to the minimum required for a valid scientific outcome and in all cases the general health and condition of an animal will remain the overriding determinant. Behavioural testing will be applied in order to reveal the disease phenotype at early stages of the disease.





41. Discovery and characterisation of genetic mechanisms affecting metabolic health and ageing

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

This project aims to exploit human and animal genetics to identify and functionally characterise new mechanisms that underpin susceptibility or resilience to metabolic disease.

Ultimately, we aim to develop new therapeutic interventions for these conditions 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?

The last 40 years has witnessed an exponential increase in the prevalence of obesity, type 2 diabetes and related metabolic conditions - in large part due to lifestyle changes. The



health and healthcare burden from these diseases has overtaken malnutrition as a leading global cause of morbidity and mortality. At the same time, advances in healthcare have markedly increased survival rates from infectious diseases and other conditions such that the ageing population has also expanded rapidly. Ageing is associated with increased risk for numerous metabolic diseases such as diabetes, cardiovascular and atheromatous disease, dementias and certain cancers. Whilst a number of frontline medicines have efficacy against some of these conditions in subsets of the population, the molecular mechanisms underpinning the adverse metabolic health effects of poor lifestyle choices and ageing across the wider population are incompletely understood and remain a pressing unmet clinical need. Ageing per se is currently therapeutically intractable. Our genes, the inherited information from our parents that determines all our bodily attributes, strongly influence our susceptibility, or resistance to metabolic disease and ageing. We have unprecedented access to ever more detailed genetic information about our populations such that medicines may soon be more individually targeted to improve therapeutic outcomes. By identifying and understanding novel genetic mechanisms underpinning metabolic disease susceptibility/resilience, this project seeks to illuminate new biology and uncover novel therapeutically exploitable gene targets discovered in human and animal models.

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

We will have identified new genes that can control how much body fat we carry when exposed to unhealthy versus healthy diets, or as we naturally get fatter with age.

We expect to publish several new studies telling us how one of the new genes we discovered that protects against diabetes works, and whether new medicines that target this gene can prevent diabetes.

We may have identified new medicines that could be further developed for the treatment of diabetes and its related diseases

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short term our project will have tested whether several newly identified genes predicted control how much fat we carry represent new ways to treat diabetes and related conditions.

In the longer term, we may be able to identify new drugs that can be used to prevent diabetes in those people who do not respond to, or have a bad reaction to, existing diabetes medicines

How will you maximise the outputs of your work?

We will publish all studies undertaken in learned journals that have open access

We will work with industrial partners to maximise the chance to develop new effective drugs that target our new genes

We will present our work at national and international conferences to get feedback and to inform our world-wide colleagues.



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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, a mouse would be housed on its own so we can accurately measure its food intake, energy expenditure (by measuring oxygen consumption and carbon dioxide production from only that individual in dedicated metabolic chambers). We would establish the normal nutrient (glucose, fats) metabolism profile of the mouse by giving an oral bolus of glucose and measuring the disappearance of the glucose over time. We would then make the mouse obese and diabetic by exposing the mouse to high fat diets for a period of time that represents poor food health durations in humans (e.g. 20 weeks; equivalent to 10 years of poor lifestyle choice in a human). We would then repeat the measures the metabolic health of the mouse as above halfway through the study (e.g. 10 weeks) to identify whether any genetic modification or medicine administration impacted early onset disease. We would repeat these measurements again at the end of the study to determine the longer term effect of genetic or pharmaceutical intervention on metabolic health.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Long term high fat diets (e.g. 20 weeks) can cause diabetes which is associated with higher levels of urination, weight loss and eventual organ failure. The strain of mice we use for the vast majority of our obesity/diabetes studies are resistant to the complications of diabetes over this time course and will be humanely culled before severe symptoms manifest.

Mice may be injected with substances (transient pain) or have surgery (string analgesic and careful recovery protocols are in place) which causes pain that is managed.

Mice with accelerated ageing mutations will become frail, develop metabolic disease and die younger than normal mice - we will scale all specifically reduced severity interventions to fit the known equivalent mean age stages (middle and old age) using previously published in depth studies on mortality rates.

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

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

The maximal severity is moderate. The proportions of animals used in each category (20% for Protocol 1; 15-20% for Protocol 2) are based upon the larger proportion of "unused genotypes" used in breeding programmes to generate necessary intermediate but



unwanted (e.g. non-experimentally useful compound heterozygote mice) mice and the smaller proportion of female mice used in experimental studies (50% of all mice generated by breeding but only a fraction of 20% (e.g. 1%) used as experimental cohorts for specific proof-of-concept on gender specificity).

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Metabolism is a highly integrated physiological processes that reflects complex interactions between the brain (e.g. appetite) the adipose tissue (storage of excess fat), muscle (calorie usage), liver (calorie storage and integration), etc. Because of this there is no way to replace the insight that investigating gene effects in whole animals provides.

Ultimately we must understand what such manipulations would do in living humans.

What was your strategy for searching for non-animal alternatives?

We use clonal cell models of several tissue types to test key hypotheses before animal experimentation is considered. For example, cultured fat cells have been used in our research to show that one 'lean gene' prevents defects in release of healthy fat cell hormones. Similar approaches are being taken with cultured human liver cells and cultured mouse muscle cells. .

Why were they not suitable?

Whilst a reductionist cell type specific approach in vitro helps us understand how each tissue might contribute to an overall effect, only whole animal studies can show how these changes affect an animals overall health and, importantly, how effective any new drug would be in the whole organism 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 use statistics (power calculations, factorial designs) based on extensive experience of our metabolic studies (for e.g. body weight, blood nutrients and hormones) to determine the minimum number of mice needed to confidently measure meaningful differences caused by gene alterations or therapy. We routinely use mice that are used commonly in



our research community and that are genetically identical to minimise variation in experiments.

We will use non-invasive technologies to determine fat mass and calorie burning capacity of mice that will allow reduction of animal numbers because we can do 'before and after' measurements (pairing of data across longitudinal studies) rather than using two separate groups of animals.

We work with other scientists with projects that allow them to use some of our post-mortem animal tissues to inform on their research on heart, blood vessels, inflammation, etc, reducing the need to use more animals in some cases.

Regular meetings of our research group ensures maximal use of our materials animals and assessment of our experimental design and statistical rigour.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We use statistics (power calculations, factorial designs; much of this consulted through local statistical experts and the NC3R Experimental Design Assistant) based on extensive experience of our metabolic studies (for e.g. body weight, blood nutrients and hormones) to determine the minimum number of mice needed to confidently measure meaningful differences caused by gene alterations or therapy. This avoids underpowered studies that are poorly reproducible (which ultimately increases animal use). We also use non-invasive technologies to determine fat mass and calorie burning capacity of mice that allows reduction of animal numbers because we can do 'before and after' measurements (pairing of data across longitudinal studies increases power) rather than using two separate groups of animals.

What other measures apart from good experimental design will you use to minimise numbers?

We outsource genotyping (identification of mice in colonies that carry the relevant mutations) to a cost-effective professional company (REDACTED) which gives us rapid control of colony management, minimising the wastage of mice across large breeding programmes.

We work with other scientists with projects that allow them to use some of our post-mortem animal tissues to inform on their research on heart, blood vessels, inflammation, etc, reducing the need to use more animals in some cases.

Regular meetings of our research group ensures maximal use of our materials animals and assessment of our experimental design and statistical rigour.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?



We use animal models best suited to address the biological question/ under study. For example, mutant obese mice are used for obesity studies. Obesity-prone 'normal' strains of mice are chosen for dietary obesity studies. Mice mutations affecting cholesterol/lipid metabolism are used in combination with high cholesterol diets that focus research questions related to atheroma as a consequence of obesity. These models are used extensively, globally, and are recognized as the mildest interventions possible whilst delivering better cross-centre replication of outcomes (e.g. obesity, atherosclerosis)

For surgical procedures, appropriate anaesthetic and pain-killers, and sterile techniques will be used. Drugs will be administered at non-toxic dosages and if unknown, this will be tested in a carefully graded dose-finding protocol.

The introduction of new non-invasive, low stress procedures for body fat mass determination allows us to minimise suffering while maximising the amount of information obtained from each animal We will use new home cage chambers that allow us to follow metabolism in real-time without interfering with the animal (Indirect calorimetry measures oxygen used/CO₂ respired). This removes the need for metabolic cages that have grid features in most studies.

We follow a path of progressive method development and refinement. For example, for nutrient metabolism exploratory methods such as oral administration of glucose with blood sampling are used to test for major effects of gene alteration on broad outcomes such as 'does diabetes improve'. Only then if an effects is clear are in depth methods used, such as using infusions of labelled nutrients (e.g. glucose) and tracking what happens to them in a living animal are employed to work out mechanisms of health change. The use of any invasive (e.g. surgical) techniques are discussed with colleagues performing similar work locally and across the country. Monitoring systems will be tailored to each model and strict humane endpoints will be applied to minimise suffering.

Training and good practice is encouraged through group meetings and regular discussions with the NVS and key staff.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The nature of complex mammalian metabolism, and its dysregulation in metabolic syndrome and ageing, cannot be adequately modelled to the level of conserved mechanistic detail required for therapeutic insight in lower organisms.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We are always looking for effective ways to improve our operating procedures (surgical and experimental) in line with best practice. We will regularly send members of our team to dedicated courses on cutting edge methods in our field that include refinement of procedures. Two examples planned for the current project include the introduction of small continuous glucose monitoring implants in mice which will obviate serial tail nicks in longitudinal studies on diabetes (high glucose). The other is the adoption of a state of the art dual catheterisation method for whole body insulin sensitivity assessment that includes, for example, infusion of washed red blood cells to maintain normal blood volume (one of the drawbacks of multiple blood sample withdrawal in some studies). We constantly strive



to improve the quality of our methods, thus generating better quality, less variable data and more optimal study power.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We follow the ARRIVE guidelines and look for any updates.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will attend the annual 3Rs conference held in REDACTED annually.

We will follow and check for updates in good practice on the 3Rs web interfaces.

Explain the choice of species and the related life stages

Mice are mammals with very similar fundamental physiology to that of humans. This is graphically demonstrated by the identical adverse metabolic and reproductive deficits found in both in mice and humans carrying mutations in the leptin gene and other well characterised genetic pathways.

Mammalian biology and the rapid generation time of mice allows timely testing of hypotheses relevant to human metabolic disease and ageing within traditional research cycles. We choose adults for studying metabolic disease because metabolic disease predominantly manifests in adulthood in humans. We choose accelerated ageing models to more rapidly identify potential anti-ageing, anti-diabetic and anti-metabolic syndrome effects as powerful initial screen that informs on the more time and cost-intensive "normal" ageing process.



42. Disease mechanisms and treatment of neuromuscular disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

No answer provided

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 is the aim of this project?

To breed, maintain and create mouse models to study neuromuscular diseases. This will provide mice for use in other protocols.

To understand the pathogenesis of neuromuscular diseases, (including Duchenne muscular dystrophy, Spinal Muscular Atrophy, Fascio-scapular-humeral dystrophy, Collagen VI deficiency, limb girdle muscular dystrophies, mitochondrial DNA depletion syndrome, Congenital muscular dystrophy), in skeletal muscle, heart and brain.

To understand the responses of muscle to injuries that mimic different aspects of neuromuscular disease (e.g. muscle fibre necrosis, muscle regeneration, prevention or reduction of muscle stem cell function).



To identify changes in gene and protein expression, or changes in activation or type of cells present, in injured skeletal muscle.

To identify factors that may improve muscle regeneration.

To investigate therapeutic approaches for neuromuscular diseases, including cell-based therapies, gene therapy, small molecule therapy and RNA therapies (i.e. antisense oligonucleotide therapies on exon-skipping, exon-inclusion or gene silencing).

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

Why is it important to undertake this work?

Neuromuscular conditions affect more than 60,000 people in the UK and an estimated 7 million people worldwide. For most of these diseases, there are no effective therapies. Examples of neuromuscular diseases include Duchenne muscular dystrophy, which has an incidence of 1 in 5,000 male births, spinal muscular atrophy which has an incidence of 1 in 6,000-10,000 births, and dystroglycanopathies which are one of the most common group of congenital and limb-girdle muscular dystrophies.

These are progressive diseases that start in childhood and have a profound impact on quality of life and mortality. They represent a significant burden for carers of affected patients and for the health care system. Several promising new treatments are in clinical trials or have recently been approved by the European Medicines Agency and/or the Food and Drug Administration for some but not all neuromuscular diseases. These new treatments include gene therapy and RNA therapies for Duchenne muscular dystrophy and spinal muscular atrophy. For the first time, these treatments have demonstrated efficacy at significantly slowing down disease progression. However, there is room for improvement in three main areas: further increase their efficacy, improve or expand the ability of these treatments to slow disease in all affected tissues (including heart and brain), and determine whether these treatments can be adapted to treat additional neuromuscular disorders. Therefore, the primary aim of our proposed project is to improve and expand current treatments. To achieve this aim, we will also need to increase our understanding of disease processes in different neuromuscular disorders. This new knowledge may lead to the identification of new treatment opportunities and interventions that could be developed into novel standalone or combinatorial treatments.

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

We hope to have a deeper understanding of disease mechanisms in neuromuscular diseases and to have determined the effectiveness of possible therapeutic strategies in different mouse models of neuromuscular disease. We will obtain more information on the molecular causes of cardiac disease and on the cognitive, emotional and behavioural problems that occur in some neuromuscular disorders.

We expect more novel experimental therapies for different neuromuscular diseases will be validated in the related mouse models, with potential of clinical translation (i.e. clinical trials initiation).

We will publish data from our work in peer-reviewed publications.



We will also present our data to relevant patient groups (e.g. Muscular Dystrophy UK, Duchenne Parent Group, Spinal Muscular Atrophy (SMA) support UK).

New treatments for neuromuscular diseases.

Greater understanding of disease mechanisms and biomarkers in neuromuscular diseases.

Development of new outcome measures for pre-clinical studies.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

We will have gained a deeper understanding of disease mechanisms in neuromuscular diseases and possible therapeutic avenues. In particular, we are working towards defining the molecular underpinnings of cardiac disease and cognitive/emotional/behavioural comorbidities in neuromuscular disorders.

In the short term (towards the end or after successful completion of our project), our data will benefit the scientific community, particularly those working in the field of neuromuscular disease.

In the medium term, our research would be of interest to the pharmacological industry, if for example, we discover a therapeutic agent that successfully treats a muscular dystrophy in one of our mouse models.

In the longer term, our work might progress to clinical trials and, if these are successful, ultimately to patient clinical benefit.

How will you maximise the outputs of your work?

We will present our data to relevant patient groups (e.g. Muscular Dystrophy UK, Duchenne Parent Group, Spinal Muscular Atrophy (SMA) support UK) and to scientists and clinicians at scientific meetings and conferences.

We will also present our work at Institute open days.

We will publish our data in high impact journals. We also intend to publish null or negative results in, for example, Wellcome Open Research.

Species and numbers of animals expected to be used

- Mice: Wild type - 1000; Immunodeficient - 500; Genetically-modified without a neuromuscular disorder - 1500; Genetically-modified models of neuromuscular disease - 7000

Predicted harms

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



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, mice will be bred and may be genotyped, usually by ear biopsy.

Some mice will be used to study treatment efficacy and disease mechanisms. Mice may be administered potential therapeutic substances by the most appropriate route (orally, or by injection). Most of these mice will not be anaesthetised, but for intracerebroventricular or intrathecal injections, mice will be under an appropriate anaesthetic. Blood samples will be taken from some of these mice. Some mice will undergo assessments to track changes in their behaviour, motor function, and/or blood pressure over time.

Some mice may be imaged to look at disease progression in specific organs, for example cardiac MRI may be performed to assess cardiac function.

Experiments will generally last from 2 weeks to 1 year.

Other mice will be transplanted with stem cells. Skeletal muscles of these mice may be irradiated or injured, as this has been shown to promote donor cell engraftment. Muscle irradiation, injury and intra- muscular and intra-arterial cell injections will be done under general anaesthetic. Regenerated muscles will be re-injured in a subset of the mice. Some mice may be exercised. Blood may be sampled from some mice. Experiments will typically last from 4-12 weeks.

We will investigate the effect of injury on skeletal muscle regeneration. Skeletal muscles of these mice may be irradiated or injured. Substances may be administered to mice, intra-muscularly, systemically or topically. Depending on the route of administration, some mice may be anaesthetised. Blood may be sampled from some mice.

Mice will then be culled for tissue collection for additional post-mortem analyses.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Some mice may experience pain as a result of surgery, but this will be controlled by the use of analgesics. To avoid pain following injection of substances, we will only inject up to the maximum recommended volumes for each injection route.

Some older mice, or mouse models of neuromuscular disease, may exhibit weight loss. We will not allow mice to lose more than 20% of their body weight.

Tumour formation can occur in aged mice. 9% of mice carrying a mutation in dystrophin develop rhabdomyosarcomas after 12 months of age. Tumours typically develop near the neck/shoulder area or hindlimbs.

Scoliosis and muscle wasting can occur in mouse models of Duchenne muscular dystrophy after 15 months of age.

If a mouse either loses more than 20% of its body weight, or it displays signs of ill health, it 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 species)?

Mild: 50%

Moderate: 50%

What will happen to the animals at the end of the study?

- 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 as we are investigating potential treatments that may affect multi-organ systems (e.g. skeletal muscle, nerves, brain, heart) and we need to optimise delivery methods in vivo and achieve efficient target-organ engagement for future clinical applications.

Disease mechanisms include interactions with the immune system, or pathologies that develop over time, that cannot be investigated in vitro.

What was your strategy for searching for non-animal alternatives?

Wherever possible, we use tissue culture to achieve our aims. Most approaches have already been optimised and tested in vitro. We are already using cell lines to test and optimise conditions for therapeutics as much as possible, before validating in vivo. For example, we test reagents such as antisense oligonucleotides in patient-derived cells in vitro as the first step. Only the lead and most efficient agents identified in vitro are selected to be tested in the subsequent experiments in animal. We are also developing human induced pluripotent stem cells and are considering organoid systems as an in vitro model.

Why were they not suitable?

Protocols for differentiation of induced pluripotent stem cells do not currently allow full maturation of the cells into cardiac and skeletal muscle tissue to faithfully reproduce disease and test therapeutics. These in vitro systems cannot replicate the complex tissue structure and cellular interactions, especially interactions with the immune system, that are a feature of the diseases we are studying. From a therapeutic perspective, these in vitro systems are insufficient to determine complex drug delivery in vivo and the efficient biodistribution required for a new drug.

Reduction

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



design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

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

We have estimated these based on the numbers of mice that we will need for each of the projects we will perform under this licence in the next five years and power calculations performed in our grant applications.

Our proposed work includes behavioural studies and muscle performance studies, that require between 10 and 15 animals/group to demonstrate statistically-significant differences, based on our power calculations.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We took advice from statisticians and used NC3R Experimental Design Assistant to determine the minimum number of mice required to achieve statistically-significant results.

When performing intra-muscular interventions, our experimental design includes using, wherever applicable, both legs, to reduce the number of mice needed to demonstrate significant differences between experimental variables.

Where possible, we will use littermates as controls.

What other measures apart from good experimental design will you use to minimise numbers?

Breeding colonies of mice will be managed to match the number required for specific experiments and replacement of breeding pairs. When genotyping, we will use the least invasive method compatible with accurate genotyping.

Wherever possible, we will use other tissues from our mice for other experiments, e.g. as controls in laboratory-based assays.

Where the effect of a specific intervention is unpredictable and the potential variation is uncertain, pilot trials using a minimum of 3 animals per group with a limited number of groups will be used to assess the value of a larger scale experiment using a range of doses with group sizes determined using power calculations. In some cases it may be possible to use a factorial design that reduces group sizes to 3-4 per group.

For experiments that require only one gender, we will wherever possible, use mice of the other gender for other gender-neutral protocols.

We are beginning to use in vivo imaging to test efficacy of approaches (e.g. cell transplantation, therapies to improve heart function), that will allow serial analysis of one mouse and thus reduce numbers.

We have purchased mice expressing therapeutic constructs transgenically (micro- and mini-dystrophin constructs used in gene therapy) to avoid the need to inject animals with adeno associated viral (AAV) vectors carrying these constructs. We are treating mice with AAV vectors only to test new constructs in a setting that best mimics clinical trial conditions.



Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

In order to achieve our objectives, we will use mice that are:

Mutant or transgenic models of neuromuscular diseases.

Genetically altered, in which either an ubiquitous, muscle specific or cell-specific promoter drives either a marker, or a potentially therapeutic, gene.

Immunodeficient (dystrophic, or normal)

Normal

Aged

Most of these mice show mild disease features, but as they get older, some of them have a worsened phenotype. Most mice will be used young, but when we use older mice, we will carefully monitor them.

When testing potential therapeutic agents, only innocuous substances would be of interest for administering to human patients suffering from neuromuscular disorders. Where appropriate, these agents will be evaluated in in vitro systems prior to applying them to mice. We will obtain guidance on doses from existing studies on humans or mice and use doses shown not to have toxic side effects. If the agent being tested is likely to have any overt toxicity, a small pilot study will be performed first. Although we cannot absolutely predict toxic effects, our checking regime will ensure that any agents causing deleterious effects will be rapidly identified. Wherever possible, we will use the least invasive effective route of delivery.

Most of the muscle injury protocols aim to mimic events that occur as a result of muscular dystrophy (muscle fibre degeneration and regeneration). We will use the most refined method for our scientific purpose and minimize as much as possible the use of the more severe injuries (e.g. cryodamage). Some agents used to induce muscle degeneration are harmful (e.g. snake venoms), but we will apply these locally to the muscle at volumes and concentrations that will not harm the mouse.

We have refined our muscle injury protocols and the age at which we apply them, so that they have the least adverse effects. We always use the most refined method that is effective for our purposes.



We have refined the breeding scheme for SMA mice from a published scheme to a new one that suits this mouse model better, based on our vast experience gained in the last several years when working on this mouse model.

We have established protocols to differentiate human induced pluripotent stem cells to undergo differentiation into skeletal and cardiac muscle cells and nerve cells in vitro. We also use expression of a myogenic regulatory factor, MyoD, to induce fibroblasts to differentiate into skeletal muscle cells. We are using these cells to test potential therapeutic approaches in vitro.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We will only use mice in this project, as the models of neuromuscular diseases that we require are mice.

We need to use mice that are at the stage of their life at which they exhibit symptoms of neuromuscular disease.

When applicable, we will use mice at a more immature life stage, (e.g newborn or young adult).

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

In experiments that involve running mice on a treadmill, we will not use electric shock, but instead will use gentle prodding to encourage the mice to run.

Motor function assessment to test endurance or forced running may cause fatigue and animals will be allowed to recover in their cages.

To minimize stress that may be caused during motor function assessment, mice will be acclimated to the equipment and procedure a week before data recording.

We will stop performing specific motor function tests if animals show fatigue and are unable to complete the test.

Following muscle injuries, appropriate analgesia will be given, as advised by our named veterinary surgeon.

Anaesthesia will be under continuous supervision and animals will be kept warm. After recovery, animals will initially be closely monitored until the mice are moving around the cage and then checked at least once daily for 2 days.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) guidelines (Wurbel 2007: NC3Rs #9 Environmental enrichment and systematic randomization Jan 2007 and Prescott 2017 Lab Anim (NY). 2017 Mar 22;46(4):152-156. doi: 10.1038/labam.1217), to ensure that our experiments are conducted in the most refined way. We will also refer to TREAT-NMD protocols for animal models of neuromuscular diseases.



We will follow the ARRIVE guidelines on reporting of in vivo experiments.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Our REDACTED sends emails to licencees on the Latest news from the NC3Rs and we consult the NC3Rs website for the latest information and guidelines. We will ensure that licencees attend any relevant courses and will implement any advances that are applicable to our work.

Explain the choice of species and the related life stages

We have chosen mouse models of neuromuscular disorders that best model disease in patients and are best suited for us in pre-clinical studies to test therapeutic approaches. The models we have chosen are mild to moderately severe. We are looking at all life stages because this protocol is interested in studying the natural course of disease progression and long term effects of treatment interventions.

We will use mice, as there are appropriate models, either already available to us, or that will become available, of the following:

Mutant or transgenic models of neuromuscular diseases.

Genetically altered, in which either an ubiquitous, muscle specific or cell-specific promoter drives either a marker, or a potentially therapeutic, gene.
Immunodeficient (dystrophic, or normal)

Normal

Aged





43. Dissecting central mechanisms of obesity and 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

No answer provided

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 is the aim of this project?

We aim to identify shared mechanisms and treatments that both reduce obesity and slow the progression of neurodegeneration.

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

Why is it important to undertake this work?

The proposed project addresses metabolic disease (e.g. obesity) and neurodegenerative disease (e.g. brain cell damage and loss), which are both major human health issues lacking broadly effective solutions. Our studies may reveal the processes that produce these diseases, which could lead to new treatment strategies. We will also test whether certain drugs can reduce obesity or slow the progression of neurodegenerative disease in mice. If these studies are successful, they would pave the way for potential treatments in humans.



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

The intended outcome of these studies are 1) increased biological understanding of the molecular and cellular pathways involved in metabolic and neurodegenerative disease, and 2) drugs that act on these pathways to reduce obesity or slow the progression of neurodegeneration, which could be advanced to pre-clinical studies.

We expect the data from the experiments covered in this license will lead to several publications in peer-reviewed journals. In addition, we will publish protocols and results, sometimes also including data on experiments that did not work, in databases that are open to the general public (e.g. protocols.io and BioRxiv).

Outputs based on our broader goals are more difficult to predict, but we intend them to have a wide and positive impact. In particular, neurodegenerative diseases and obesity contribute to more than a third of the total burden of disability-adjusted life years (DALYs) in the elderly population. By shedding light on the processes in the brain that may lead to metabolic disease (e.g. obesity) or damage to brain cells (e.g. neurodegeneration), we may contribute to the future development of more effective treatments for these major diseases.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Diet and neurodegeneration: In the short-term, our studies would prompt us to isolate the relevant dietary or metabolic factors that affect the neurodegenerative progress. In the longer term, these findings could affect how individuals at risk for neurodegenerative disease might alter their lifestyle to reduce their risk.

Drug treatments for neurodegeneration: In the short-term, we aim to understand how some drugs given to treat diabetes and obesity (e.g. Liraglutide) also improve brain function in Alzheimer's disease and Parkinson's disease. In the longer term, we would test additional drugs and additional mouse models of neurodegeneration, or even test promising drugs in humans suffering from neurodegeneration.

Drug treatments for metabolic disease: Our plans to test whether drugs that act on cultured human brain cells also affect food intake and body weight will test how well cultured cells can predict effects on body weight, and may reveal new ways in which food intake is regulated. In the longer term, these drugs or drug combinations could be tested in humans, especially since we will preferentially work with drugs already approved for use in humans.

Central mechanisms of metabolic disease: In the short-term, these studies will explore and identify genes that act in the brain to regulate food intake in order to link genes associated with obesity and behavior that contributes to obesity. In the longer term, drugs targeting these genes might lead to new treatments for obesity and diabetes.

How will you maximise the outputs of your work?

Our research work will be carried out in collaboration with research teams who have years of experience in working with mice that develop neurodegenerative disease. All our research findings will be made available to other scientists through presentations at



scientific conferences and publication in peer-reviewed journals. In addition, protocols developed for this work will be freely distributed to the community (e.g. Protocols.io). Negative results may be published on open-access servers (e.g. BioRxiv). Data will be stored in electronic format wherever possible and stored for at least 10 years to enable our group and collaborators to easily retrieve and build on past results, or reanalyse data using new methods.

Species and numbers of animals expected to be used

- Mice: 19,000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mice will undergo a brief surgical procedure which involves injecting infectious proteins (Prions) into the brain to cause brain damage (neurodegeneration). About a week later, mice will be given food that is high in fat and/or carbohydrates, which they like to eat so much it makes them fat. Following this, mice will be given experimental drugs to reduce obesity, or reduce the signs of neurodegeneration. The effect of drugs on obesity and/or diabetes will be analysed by measuring their food intake and activity along with body composition imaging to find changes in body fat. Blood samples may be taken to measure hormonal changes in the blood and mice may also be exposed to a brief test similar to one used in humans to see if their diabetic state has been reversed. To analyse the effects of the drug treatment on neurodegeneration, mice will be tested for changes in behavior (e.g. burrowing pellets of food) and the amount of certain proteins in the post-mortem brain tissue. Experiments will typically take 4-24 weeks.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Prion-injected mice will develop defects in their movements and memory at a predictable time after injection, unless they are killed sooner. In case of obese mice, these defects may appear sooner. All prion-injected animals will be regularly monitored for onset of early signs of clinical disease. Once these signs are observed, mice will be inspected daily to determine if additional signs of the disease emerge, and twice daily if prion signs are progressing quickly. We will monitor the general condition of the animal and pay specific attention to breathing, coordination, posture and gait. All signs will be documented on clinical observation sheets. Mice will be killed as soon as clinical prion disease can be diagnosed.

Most animals will experience transient pain or discomfort due to drug administration, which will be treated with appropriate anaesthesia/analgesic.

Mice fed with modified diets will exhibit long-lasting increase in food intake (hyperphagia) and obesity which may cause type 2 diabetes. These conditions may be reversed in the presence of therapeutic interventions as a part of the experimental design.



Food restriction and insulin tolerance tests may cause a drop in blood sugar (glucose) levels (hypoglycaemia), which will be treated by either giving mice food, or directly giving them glucose. Mice will be killed if hypoglycaemia is not reversed within two hours of these interventions.

Mice that undergo surgery (prion injection, osmotic mini-pump implantation and guided injection into the brain) may develop complications such as failure to eat and/or to move normally, or failure of the wound to heal normally. In these cases, the NVS will be consulted. In rare cases (expected < 2%), animals may experience wound breakdown or infection, which will be treated and wounds re-closed within 48 hours post-surgery.

Treatment with substances (e.g. drugs or viruses) targeting genes and cell types important for regulating metabolism are expected to cause a change in food intake, body weight and body condition score (observed as a readout of the experimental design as well as a measure of animal health). Mice will be killed if their body weight drops more than 15% below the weight of age-matched and sex-matched control mice or if they show other clinical signs or deterioration in body condition score.

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

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

The expected severities are mild (approximately 30% of mice) and moderate (approximately 70% of mice).

What will happen to the animals at the end of the study?

- 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?

Metabolic and neurodegenerative disorders affect millions of people worldwide. These diseases are mostly untreatable, and represent a large, unmet clinical need that cause a substantial economic impact. These diseases may have shared causes, which are still poorly understood, but likely interact at the level of the whole animal rather than a single cell type. For example obesity leads to increased blood pressure and activation of the immune system, which are thought to independently contribute to neurodegeneration. As such, it is essential to use animal models to understand which of these factors are most relevant, and to test possible treatment options.

We work extensively with cell culture systems to understand the behavior of individual cell types that contribute to metabolic and neurodegenerative disease. However, due to the complexity of the brain and the lack of culture systems that can fully mimic this complexity, animals remain irreplaceable. Furthermore, animals provide insight into the clinical,



behavioral, physiological and neuropathological outcomes that can lead to development of better therapies.

What was your strategy for searching for non-animal alternatives?

The proposed work cannot be readily replaced with non-animal alternatives, however:

wherever practical, we will use our cell culture systems to prioritize drugs that act on specific brain cells before carrying out targeted animal studies

wherever practical, we will use our cell culture systems to prioritize genes that act on specific brain cells before carrying out targeted animal studies

where possible, we will use human tissue samples to confirm the significance of key target genes in human neurodegenerative disease and obesity

Why were they not suitable?

Although brain cell culture systems provide useful insights into obesity and neurodegeneration, they cannot completely replace the need for animal studies. Specifically, cell culture models do not mimic complex interactions between cell types and body systems that likely contribute to human disease. They also do not allow measurement of behaviors or physiological outputs relevant to disease, making it difficult to predict how results obtained from cell culture systems will be relevant to human disease progression or treatment. Therefore, we will conduct both cell culture and animal experiments to identify the most promising strategies for treating obesity and neurodegenerative disease.

Reduction

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

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

We will use the minimum number of mice needed in all experiments for reproducible results and statistical validity in line with the ARRIVE guidelines www.nc3rs.org.uk/ARRIVE. We will maximize the use of each experimental animal by maximizing the readouts obtained from each animal thereby reducing the number of animals needed. For most of the quantitative experiments, we will determine the sample size required based on relevant literature. Otherwise, statistical power analysis will be used to set sample sizes (significance level of 5%, power of 80%).

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We have referred to the PREPARE guidelines (<https://norecopa.no/prepare>) and the NC3RS Experimental design assistant (<https://www.nc3rs.org.uk/experimental-design-assistant-eda>) to help plan and design our experiments. We have considered the number of independent experiments that will be performed when calculating total animal numbers



and have consulted the “Guiding Principles for Behavioral Laboratory Animal Science” paper to ensure that our experimental design incorporates the most appropriate tests.

What other measures apart from good experimental design will you use to minimise numbers?

The following steps will be taken to ensure best breeding practice to efficiently supply mice for research while minimizing animal wastage:

We will ensure high standards of animal care and welfare and the breeding of animals will be conducted according to the best practice.

Dosage and safety of all drugs and drug combinations will be validated by a pilot study.

Where possible, we will use published data to inform safe dosing limits and adverse effects of drugs, thereby reducing the number of animals needed for screening and testing.

We will use cell culture systems to prioritize and select drugs and genes that act on specific brain cell populations of interest before carrying out targeted animal studies.

Mice will be grouped into experimental cohorts large enough to allow sufficient numbers for sampling at regular intervals.

We will randomly assign mice to experimental groups and where possible, we will conduct blinded studies to minimize bias.

Suitable experimental controls will be used for each study, based on the study design and specific aims.

We will maintain minimum colony sizes that are required to meet the demands of our research experiments and avoid overproduction.

Detailed breeding records will be kept, enabling the selection of the most appropriate breeding stock.

Where appropriate, genetically altered animals will be used to reduce the number of animals required to achieve specific aims.

Cryopreservation of genetically altered mouse lines will reduce animal wastage and costs associated with mouse colony maintenance.

We will ensure that researchers and technicians working on this project are appropriately trained and competent to maximize the success rate of experiments and thus minimize the number of animals used.

Detailed study plans describing the aims and objective of each experiment along with the experimental steps, treatment groups and sizes and methods for data analysis will be written down to guide 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Models:

Mouse models of prion disease are superior to those available for other human neurodegenerative diseases since they show many of the same features of human prion disease, including the loss of brain cells that are not seen in other mouse models. Prions are essentially infectious proteins. They have a particular three-dimensional structure and can cause normal proteins in the brain to fold into that same structure, causing a “chain reaction” of misfolded protein. Therefore, injecting normal mice (e.g. C57Bl/6J) with prions from mouse causes misfolded proteins to spread throughout the brain leading to brain cell damage and loss. Injected mice develop clinical signs of prion disease (ruffled fur, rigid tail, hunched posture, loss of coordination and balance) after about 4-5-months in a manner that has been described in detail and that is very consistent between individual animals. This model aids better understanding of the how neurodegenerative disease progresses, and how treatments might delay or slow disease progression.

Genetically altered REDACTED will enable us to more easily count connections between brain cells to determine how they change with metabolic or neurodegenerative disease. Similarly, genetically altered REDACTED will be used to facilitate change in gene expression in specific brain cell populations and evaluate the gene function with respect to metabolic and/or neurodegenerative disease.

We may use genetically altered mouse models of obesity (and/or other metabolic diseases associated conditions such as type 2 diabetes) or models that are resistant to obesity (and/or other metabolic diseases associated conditions such as type 2 diabetes), typically on a C57BL/6J genetic background. For e.g. we may use Melanocortin-4 receptor (MC4R) knockout mouse model that exhibits obesity, hyperphagia, hyperglycaemia (excessive levels of blood glucose) and hyperinsulinemia (excessive levels of blood insulin). These models will enable us to identify the effects of metabolic associated genes on neurodegenerative disease progressions and/or therapeutic interventions.

Diet-induced models of obesity and metabolic disease are well-established in the field, and complement existing and emerging genetic models. Specifically, the time-course of diet-induced obesity in C57BL/6J mice and the accompanying changes we intend to measure (e.g. body weight, body composition, food intake, energy expenditure, hormone levels, and glucose homeostasis) are all well-described in the literature. These mice serve as useful tools in evaluating the effects of anti-obesity compounds and therapies. We will also use them in combination with prion infection to study the links between metabolic disease (and substances used to treat metabolic disease) on neurodegenerative disease progression.

In drug-treated diet-induced obese mice with or without prion infection, we hope to see reduction in body weight and related symptoms, but it is possible that there may be unintended side effects such as anxiety or difficulty moving. We will therefore monitor mice regularly for potential adverse effects. We will use body-condition scoring charts following published standard criteria rather than solely relying on weight changes, since weight loss in an obese mouse may actually indicate improved health rather than deteriorating health.



Implementation of body condition scoring, regular body weight measurements, and observation charts for neurological signs and general welfare will allow us to accurately monitor the neurodegenerative process and define humane endpoints to reduce suffering.

Humane endpoints have been chosen to ensure that animal suffering is minimised whilst obtaining the most valid scientific output. Most prion-infected mice will be killed at early time points during disease progression, well before they develop prion disease. Tissue collected at these time points will be analysed for histological and biochemical readouts of the disease state; such as neuronal loss, detection of PrP^{Sc} (disease-associated form of the prion protein) and markers of inflammation. However, a cohort of mice will be allowed to develop clinical signs of prion disease to assess how metabolic disease and therapeutic interventions affect the later stages of neurodegenerative disease progression. In rare cases (estimated < 5%), mice left to develop clinical signs of prion disease may exhibit rapid disease progression. However, this is very unlikely since prion disease can be confidently diagnosed at relatively early stages by the appearance of early indicators such as decreased motility and tail rigidity, and confirmatory signs such as ataxia and loss of righting reflex. Therefore, suffering is limited by the daily observation and monitoring of these signs.

Methods:

All procedures (breeding transgenic mice, prion inoculation, and administration of drugs, behavioral and physiological analyses, and guided viral injections) will be conducted based on previous publications.

Animal suffering will be minimised by:

performing all surgical procedures (prion inoculation, osmotic pump implantation and guided brain injections) under general anaesthesia and providing analgesia (painkillers) before and/or after surgery.

minimizing the number of surgical interventions: most animals will receive prion inoculation followed by one or two osmotic fusion pump implantations (with at least 4 weeks in between subsequent surgeries). Surgical implantation of telemetry device will be carried out at the same time as osmotic fusion pump implantation. In other experiments, mice will receive one set of brain injections on either side of the head (at most two injections, one on each side of the head) on a double transgenic background. For embryo transfer, preference will be given to non-surgical method whenever possible.

giving mice pain relievers where appropriate and administering substances (e.g. drugs) via the least invasive route that is practical. All administration protocols adhere to published guidelines, LASA Good Practice Guidelines, and as advised by the NC3Rs. For most drugs, daily dosing will be required for a period of 50-60 days, corresponding to the onset of clinical signs or defined endpoints. Wherever practical, mice will be given these drugs via implanted osmotic fusion mini-pumps rather than daily injections. In case of daily injections, mice will be monitored for adverse reactions and body condition (as standard for all dosing studies). Pain relief may be given as recommended by a Named Veterinary Surgeon (NVS)/ Named Animal Care and Welfare Officer (NACWO) if mild signs of discomfort are noted. Dosing will be stopped and the NACWO/ NVS/ Home Office Inspector (HOI) contacted if moderate signs develop.

using the most refined compounds identified from the literature, our cell culture work, or from drug libraries. After identifying the dosage routes, frequency and concentration, the



minimal likely effective dose will be used. In case of new compounds or combinations, small pilot studies will be conducted in mice to provide this information.

using ultra-fine needle syringes, such as Hamilton Neuros™ syringes for stereotaxic injections, therefore reducing injection site damage.

group housing mice with enriched environments as much as is practical.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We are unable to use animals that are at a more immature life stage/less sentient/terminally anaesthetised due to the following reasons:

To study the complex interrelation and course of disease progression (obesity and/or neurodegeneration), a multi-organ living organism with a developed brain that can closely mimic human disease needs to be utilized as an appropriate model.

In order to understand the mechanism of disease progression and identify therapeutic strategies, we need to analyse brain structure and animal behavior that cannot be obtained from immature or terminally anaesthetised mice.

Less sentient species cannot provide accurate insight into the human disease progression as they lack sufficient similarity to humans.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Where permissible by the experimental strategy, mice will be group housed in small numbers (3-4 per cage) and will be provided with environmental enrichment wherever practical. Male mice may exhibit aggression and therefore will be regularly monitored for the presence of fight wounds. Any mice with evidence of fighting will be closely monitored and animals may be separated if fighting continues. The NACWO/NVS will be informed and analgesia may be given as directed by the NVS. If an animal sustains injuries from fighting that are noted to cause distress and suffering and that cannot be treated with topical or systemic therapy (as directed by the NVS), it will be promptly killed by Schedule 1.

Post-surgery (Prion inoculation, osmotic mini-pump implantation, guided brain injection), mice will be monitored daily for a minimum of 72 hours and maybe given appropriate care (analgesic for pain, re-closing of uninfected and minimally inflamed wounds).

For experiments requiring short-term single housing or transfer to a different type of cage (e.g. metabolic cages), mice will be allowed to acclimatize to the individual housing and/or the new cage for minimum period of 24 hours.

Body weight, body condition scoring, glucose and ketone levels will be measured regularly (daily to weekly) to assess animal health. Mice exhibiting poor tolerance to fasting may be re-fed or given an intra-peritoneal dose of glucose.



Mice will be regularly (daily/weekly) assessed for any changes in their motor function (e.g., disturbances of gait and abnormal posture or muscle tone), level of arousal (e.g., hyperactivity and lethargy), and psychological status (aggression, biting, licking).

All prion-inoculated animals will be regularly monitored for onset of early indicators of clinical disease. Once noted, mice will be inspected daily thereafter for clinical confirmatory signs of the disease and twice daily if prion signs are progressing quickly. The general condition of the animal will be assessed with specific attention given to breathing, coordination, posture and gait. All signs will be documented on clinical observation sheets.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow the LASA Good Practice Guidelines and PREPARE guidelines to ensure all our experiments are conducted in the most refined way. We will refer to the NC3Rs (<https://www.nc3rs.org.uk/3rs-resources>) and use our REDACTED biomedical services search tool REDACTED to stay informed and continuously refine our experimental strategies. We will consider the number of independent experiments that will be performed when calculating total animal numbers and have consulted the “Guiding Principles for Behavioural Laboratory Animal Science” paper to ensure that our experimental design incorporates the most appropriate tests.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

During the project, we will regularly follow updates on the NC3Rs and RSPCA websites specially pertaining to the 3Rs and animal welfare. Experimental strategies will be modified to incorporate new technologies and approaches. We will ensure our participation in review meetings held in our establishment to discuss validity and usefulness of our mouse models and review animal wastage. We will stay updated on scientific literature in our field which would enable us to further refine our experimental approach.

Explain the choice of species and the related life stages

We intend to understand how genes, cell types, dietary factors, and drugs contribute to the development (or treatment) of metabolic disease (e.g. obesity) and neurodegeneration. To understand how these contributing factors are related, it is essential to use animals where behaviors related to obesity (e.g. food intake) and neurodegeneration (e.g. defects in memory) can be studied. Mice serve as the most appropriate species to use for these studies since mice and humans share more genes, cell types, and behaviors than flies, worms, or fish that are commonly used in research. While in theory monkeys would be even more similar to humans, mice have been used more extensively so obesity and neurodegenerative models in mice are very well established. Furthermore, there are many genetically altered mice that will aid our studies to understand the details of how obesity and neurodegeneration is caused, and how these diseases might be treated.

Since obesity and neurodegenerative disease largely affect adult humans, we will perform our studies in adult mice. Specifically, most of our studies will be carried out using young or adult mice (males and/or females) to enable the study of metabolic and/or neurodegenerative disease progression and the effect of interventions (metabolic and environmental factors, agents (e.g. drugs), and genetic). Prion infection in young mice results in the development of clinical signs of prion disease after about 4-5 months. Their disease onset and progression is well-described in scientific articles and is consistent



between animals, enabling us to compare the effect of diet or experimental agents (e.g. drugs) on disease progression.



44. Drug discovery in neurodegenerative diseases

Project duration

5 years 0 months

Project purpose

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

Key words

dementia

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Neurodegeneration leading to dementia is a major unmet medical need and patient numbers are expected to increase with the increase in the aging population. Currently no therapies can slow or reverse the disease process. This work will advance the discovery of new therapies, by providing support for evaluation of the uptake, metabolism and efficacy of novel potential drug candidates in rodents.

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

What are the potential benefits that will derive from this project?

The benefits to humans are the potential identification of new molecules which may become clinical candidates for the treatment of neurodegenerative diseases. This work will also advance science; testing of selective drug molecules targeting specific proteins thought to be involved in the disease process will allow researchers to understand better the role of these proteins in the disease state.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?



Over the course of these 5 years, we will use both mice and rats. We anticipate using a maximum of 7000 mice (adults and neonates) and 1500 adult rats.

Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The likely maximum level of severity is moderate and most procedures will be mild.

Following surgical procedures most animals will experience moderate pain and distress. Anaesthesia and analgesia will be established and maintained at sufficient level for the animal not to feel pain throughout the entire procedure.

Following injections, animals will experience transient mild pain

All animals will be observed by trained staff to check for sign of distress such as:

appearance

behavioural abnormality or distress

weight loss

alteration in their natural behaviour and interaction with other animals

Appropriate remedial action will be taken and the following endpoints used when necessary to limit suffering: where anaesthesia is required, animals will be monitored and housed separately from others until fully recovered; more frequent monitoring; placing on a heated blanket; if the condition deteriorates and/or the severity is likely to exceed moderate, animals will be culled by a Schedule I method.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

For all work, animal use will be replaced where possible with in vitro alternatives – this will include the use of cell culture models (primary and stem cell derived) and in vitro models of drug metabolism. Only compounds with an acceptable profile in the in vitro assays will be taken into in vivo models.

For drug uptake and metabolism (pharmacokinetic) studies, animal use is essential as it is important to measure drug exposure in an intact system.

For in vivo efficacy studies, we will measure activity on biomarkers and disease progression. For both of these measures, we require the use of animals. Many processes are involved in neurodegeneration, and to ensure that drugs tested are likely to be of benefit we need to determine the efficacy of the potential therapy in the complex disease state rather than a simplified culture model.



Reduction

Explain how you will assure the use of minimum numbers of animals.

Prior to the use of animals, we will test all potential drug candidates in cell culture assays. These will include assays to determine the potency of the drug compound at its molecular target and the selectivity of the compound for the target. We will use biochemical and cell assays to determine that the compound is stable and likely to enter the brain before dosing to live animals to ensure the compound is likely to reach sufficient concentration to have an effect, but not so high as to cause adverse events. We will also test the compound in cell culture models of neurodegeneration prior to use in animals.

Experiments will be designed in consultation with a statistician to minimize the number of animals required. Sample size will be predetermined based on a high statistical power and based on previous experience. Together these measures will decrease the numbers of animals used, by ensuring that only those compounds most likely to test the hypothesis are selected for in vivo work, minimising the need to repeat experiments.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Our work will predominantly take place in mice. The majority of animal models of neurodegeneration are based on genetically modified mouse models; the refinement and understanding of these models has advanced in recent years. For each potential drug target, we will select the most appropriate genetically modified model based on the literature, and will select the timepoint for dosing based on biomarkers of disease.



45. Effects of radiotherapy on lung injury

Project duration

2 years 0 months

Project purpose

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

Key words

pneumonitis, radiotherapy

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 is the aim of this project?

The overall aim is to test whether low doses of radiotherapy to the lungs can reduce the severity of lung injury by reducing the immune response to lung damage.

The immediate aim of the project is to generate laboratory data that is urgently required to help design clinical trials of low dose lung radiotherapy in patients with severe COVID-19 infection.

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

Why is it important to undertake this work?

The COVID-19 pandemic has directly affected over 1 million people to date and the number of deaths is rising rapidly. A subset of COVID-19 patients experience severe acute respiratory distress syndrome (ARDS) which appears to be caused by an intense immune and inflammatory response to the primary lung infection. There are currently no effective treatments for this phase of the disease and 50% of patients requiring ventilation will die. Low dose radiotherapy to the lungs has historically been used as an effective treatment for pneumonia, and the immune-mediated nature of COVID-19 related ARDS indicates that it



might have value in this disease. However, the optimum dosing and scheduling are unknown, and the immune mechanisms involved are unclear. The clinical community is developing a clinical trial of low dose radiotherapy for severe COVID-19 and has urgently requested pre-clinical data to help decide how best to give the radiotherapy - what dose, how often - and to help identify which patients would benefit.

The proposed work will provide unique and urgently needed information that will increase the likelihood of developing an effective new treatment for COVID-19.

The project will also generate new understanding of the impact of low dose radiotherapy on immune processes within the lung, which may provide benefits for patients with a wide range of inflammatory lung diseases.

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

We will generate:

new understanding of how low dose radiotherapy modifies immune responses in the lungs

new information on the best way to deliver radiotherapy to the lungs to reduce the severity of lung injury caused by bleomycin (for example: when to treat, how often, what dose to give, which organs to treat)

this information will be published in peer-reviewed journals and made available to the international scientific and clinical communities

our findings will be used in real time to influence the design and delivery of clinical trials testing the use of low dose lung radiotherapy for severe COVID-19 illness

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

We are optimistic that the following groups of people will benefit from our findings in the timescales shown:

patients with severe COVID-19 infection; within 3 - 6 months

clinicians developing clinical trials for severe COVID-19 infection; within 3 - 6 months

scientists and clinicians researching and treating acute respiratory distress syndrome caused by other infections or exposures; within 6 - 12 months

How will you maximise the outputs of your work?

I am already part of a highly active international consortium of radiation oncologists and radiation biologists who are working together to develop clinical trials and increase our understanding of the effects of low dose radiotherapy on acute respiratory distress syndrome. I will share our new knowledge with this community, making use of fast-track publication systems such as bioRxiv. The consortium will also provide timely, expert advice and objective criticism of our experiments and data. With the suspension of the national and international conferences that would usually be used as a forum for



dissemination of data, we will utilise webinars and other remote communication platforms to present and share data.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mice will typically inhale bleomycin through their nostrils while under brief general anaesthetic. Some mice will experience rapid breathing after this, but only for a short period of time. Some mice experience lethargy after the treatment but this usually lasts for less than 2 days, after which time they recover and show normal behaviour.

Some of the mice will receive targeted low dose radiotherapy to their lungs and other organs including the spleen. This treatment is given while the mice are under general anaesthetic and the radiotherapy doses are extremely low, so we don't expect the mice to experience any side-effects from this treatment. Most mice will receive a single radiotherapy treatment but some may receive up to three treatments, to enable us to see if repeated treatments are more effective. The total dose of radiotherapy is still extremely low so we don't expect any side-effects from multiple treatments.

At various times during the experiment we may perform CT scans or MRI scans of the mice to see whether the lungs are inflamed. These tests will be done with the mice under general anaesthetic and will not cause any side-effects.

We will take small blood samples from mice every few days, typically four times and up to a maximum of eight times. These will cause very brief and mild pain but will not have any lasting side-effects.

About two weeks after the bleomycin treatment, the damage to their lungs may cause some mice to lose weight and show signs of lethargy. Any mice losing more than 25% of their bodyweight will be killed humanely to avoid suffering.

All our experiments will last a maximum of 28 days. At this time, any mice who are still alive will be killed humanely and their blood, lungs and other tissues analysed in detail so we get as much information as possible from every mouse in the study.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The initial increase in breathing rate can last up to one hour.
The early symptoms of lethargy can last up to 48 hours.
Pain caused by blood tests will last a few seconds.



Weight loss and lethargy caused by lung inflammation can be progressive over several days. Increased breathing rate may be progressive over several days. Mice may be subjected to repeated anaesthesia over the course 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 species)?

Based on published data, we anticipate 50% of bleomycin-treated mice to develop more than 25% weight loss within the 28 day experimental period. These mice will be humanely killed.

Increased breathing rate occurs transiently after bleomycin administration in 90-100% of mice but usually resolves within one hour. Increased breathing rate may also be observed in some mice at later stages but this is not a common feature of the model and weight loss tends to occur before breathing is affected.

Similarly, lethargy and reduced interactions with other mice may occur in some mice but this is not a common feature and weight loss tends to occur before these symptoms become apparent.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

An international consortium of radiation oncologists is planning an urgent clinical trial to test whether low dose radiotherapy to the lungs can improve outcomes for patients with severe COVID-19 infection. The consortium has requested information on how best to give the radiotherapy - what dose, how often, at what stage of the disease, and to which patients. It is only possible to get this information by testing the effects of different doses and schedules of radiotherapy on mice with acute lung injury.

What was your strategy for searching for non-animal alternatives?

We will be doing additional laboratory experiments on immune cells taken from human subjects and grown in the laboratory, but these will not be able to provide the critical information needed for the clinical trial.

We are optimistic that clinical trials of low dose lung radiotherapy for COVID-19 lung disease will be up and running within the timecourse of our project. I am part of the consortium overseeing these studies and will have access to early results. If appropriate, we will use emerging clinical data to refine the research questions we are asking in our animal studies. This may reduce the number of experiments required, and will make our results more relevant to the clinical questions.



Because acute lung injury is a complex and dynamic process involving multiple tissues and cell types, it is difficult to replicate it with a non-animal alternative. However some research groups are developing non-animal models that use lung slices; these are showing some promise so we have considered whether we could use them.

Why were they not suitable?

As well as lung injury involving multiple cell types, the effects of low dose radiotherapy on the immune response involve effects on multiple tissues and cell types including lung tissue, circulating blood cells, lymph nodes and spleen. The effects of radiotherapy on lung slices would therefore not be representative of the effects of whole lung radiotherapy. We therefore believe that experiments in live mice are required for us to properly test whether low dose radiotherapy can reduce inflammatory lung injury.

Reduction

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

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

We estimate needing to use approximately 30 mice in each experiment so that we can have confidence in the results. We estimate needing to perform about 20 experiments in order to test all the different radiotherapy doses and schedules as well as the testing whether giving radiotherapy to the spleen and the heart affects outcomes.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We will perform two pilot studies: the first will establish the best dose of bleomycin to use to cause lung injury that resembles severe COVID-19 infection. The second will determine how consistent the effects of bleomycin and radiotherapy are on lung inflammation in mice. The results will tell us how many mice we will need to include in all the subsequent experiments in order to give us reliable results.

If we find that mice treated with bleomycin and no radiotherapy all behave in a very similar way, we will use the results of the first experiments as a comparison for the all the subsequent experiments (with different radiotherapy doses, for example).

We will keep abreast of early outcomes of clinical trials testing low dose radiotherapy in COVID-19 patients and if appropriate will adapt our experiments in order to ask the most important research questions or to take account of new clinical information. This may reduce the number of experiments needed to answer the key research questions and hence reduce the number of animals used.

What other measures apart from good experimental design will you use to minimise numbers?



By taking blood samples at regular intervals and undertaking CT or MRI scans of the mouse lungs, we will obtain multiple pieces of information from every mouse. This will increase the data generated by each experiment, which may enable us to reduce the number of mice used. Also by obtaining and analysing multiple samples from each mouse at the end of the experiment (lungs, blood, fluid from the lungs) we will increase the amount of information for each mouse which may enable us to reduce the number 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will treat healthy mice with a chemical called bleomycin that causes inflammation and injury to the lungs, which is very similar to the lung injury seen in patients with severe COVID-19 infection. Mice treated with bleomycin experience minimal side-effects in the first two weeks after treatment, and then may start to lose weight and become tired as their lungs become scarred. We will humanely kill any mice that start to develop these symptoms in order to cause the least suffering possible. This method will enable us to test whether low dose radiotherapy reduces the severity of lung injury caused by bleomycin, without causing distress or lasting harm to the animals.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We need to use adult mice because the effects of bleomycin on the developing lungs of less sentient animals are very different from the effects on adult lungs. We can't do these experiments on mice who have been terminally anaesthetised because it takes two to four weeks for the effects of bleomycin to be seen.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Mice will be housed in group cages (five per cage) to enable social interaction. Because we anticipate weight loss, mice will be introduced to a soft diet prior to starting experiments so that they become accustomed to it and will find it easier to eat at later timepoints.

Most of the procedures will be done under general anaesthetic in order to minimise harm and suffering.

Mice will be monitored and weighed on a daily basis from the start of the experiment, and a structured checklist will be used to look for the expected symptoms and signs of the bleomycin model as well as signs of general wellbeing. If mice start to display signs of severe lung injury (weight loss, lethargy) they will be humanely killed.



What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Our project has been designed in accordance with the PREPARE guidelines and we will use the NC3R's Experimental Design Assistant (EDA) to optimise our experiments. We have sought advice from internal and external experts in the field of inflammatory lung injury to ensure our specific experiments are as refined as possible. We are active participants in REDACTED and are constantly updating our radiotherapy protocols and quality assurance practices to ensure that radiotherapy is delivered as accurately as possible in order to avoid harm.

The results of our experiments will be reported according to the ARRIVE guidelines.

In the conduct of our experiments we will adhere to the Institution's Animal Research Policy.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We are continuously updating our research practice through reading the scientific literature, attending seminars and webinars and submitting our research findings and proposals for critical expert review. The work proposed in this application will undergo detailed critical and ethical review when we submit it for funding.

We will monitor ongoing developments in the use of lung sections as models of lung injury and will seek to collaborate with researchers who are using and developing them if this will improve the quality of our research and reduce the number of animal experiments required.

We regularly consult the NC3Rs website and have used it to access the PREPARE and ARRIVE guidelines and the Experimental Design Assistant (EDA). These tools have been used to design and enhance our project, as described below.

Explain the choice of species and the related life stages

We are using adult mice for these experiments because we know how they will respond to the bleomycin treatment and we know that the effects of bleomycin on the lungs of these mice are similar to the effects of severe COVID-19 infection in patients.





46. Elephant endotheliotropic herpesvirus (EEHV) clinical vaccine candidate trial

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Asian elephant (<i>Elephas maximus</i>)	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 is the aim of this project?

The aim of this project is to determine the safety and efficacy of a candidate vaccine against EEHV.

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

Why is it important to undertake this work?

EEHV is an important pathogen affecting captive and wild populations of the endangered Asian elephant (*Elephas maximus*). The virus can cause a highly fatal haemorrhagic disease in juvenile elephants (Long et al., 2015; Zachariah et al., 2007). Treatment of viraemic elephants with antiviral drugs has not proven effective and is compounded by the rapid progression of the disease with death often occurring within hours to days after the first clinical signs are detected (Richman et al., 2012). Thus, this work is important to develop a vaccine candidate and vaccination protocol that can be applied to protect Asian elephants to prevent developing lethal EEHV-HD. A sufficiently protective EEHV vaccine



will reduce the mortality in elephant populations. This directly supports animal welfare of the herd (as the loss of infants is highly stressful) and supports the aim to create sustainable populations.

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

The primary expected benefit is the creation of a vaccine candidate that is safe and efficacious to induce an immune response.

The work is expected to provide information on EEHV vaccine targets.

The findings obtained in the project will be published in peer reviewed journals and presented at scientific conferences and meetings.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Production of an effective vaccine candidate against EEHV will benefit animal welfare by reducing the incidence of clinical disease in Asian elephants. The full benefit is likely to be realised after the end of the project when vaccination can be implemented into veterinary practice.

New insights into the quality and quantity of immune responses elicited by selected vaccine candidate antigens and host-pathogen interaction will be valuable to the scientific community working on Asian elephants and evolutionary related species.

How will you maximise the outputs of your work?

New findings will be presented in peer reviewed journals and at national and international scientific conferences.

Species and numbers of animals expected to be used

- 6

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will be vaccinated up to four times and in this context and around one week thereafter blood samples will be drawn for laboratory analysis of the effects the vaccine induces.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.



The vast majority of animals will show no adverse side effects. A small number of animals might display mild and transient adverse effects, such as local swelling. These will be addressed with husbandry and veterinary support and will not lead to a lasting effect.

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

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

All animals are likely to experience only mild levels of severity.

What will happen to the animals at the end of the study?

- 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?

Due the specific nature of EEHV infections no alternative models are available.

What was your strategy for searching for non-animal alternatives?

Non-animal/in vitro models are not available.

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 experiments are designed to ensure the appropriate number of animals are used: the numbers selected reflect the minimum that enable robust experimental design compatible with obtaining reliable and meaningful results.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?



We have sought advice from local statisticians, but also consulted the regulatory authorities for licensing vaccines and veterinarians with experience in early clinical studies.

What other measures apart from good experimental design will you use to minimise numbers?

This is itself a pilot clinical study and materials obtained and left after analysis will be stored for further analysis 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The approach selected uses established vaccines approaches that do not show lasting suffering or in any tested mammalian species. The procedures to apply the vaccine and sample blood are equally well established and cause minimal suffering and distress only.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

There are no model species that could be used, nor are there life stages less sentient that would apply to be used.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The animals will be trained for the application of vaccines and blood withdrawal. The animals will be closely monitored after all treatments and if required pain management against local swelling will be provided.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

OIE (World Organisation for Animal Health) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals

Home Office The Harm–Benefit Analysis Process _Advice note

Home Office Guidance to Animal (Scientific Procedures) Act 1986

How will you ensure you continue to use the most refined methods during the lifetime of this project?



We are in constant exchange with other groups working on EEHV and stakeholders so that we will hear about advances regarding the particular disease and impact this can have on our approach. We are also constantly reviewing progress in vaccination to inform us how we can improve procedures.

Explain the choice of species and the related life stages

The choice of animals is determined by the lack of any in vitro or in vivo model. Adult animals are suitable to determine the safety and efficaciousness of the approach.





47. Embryonated Eggs for Diagnosis and Research

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

No answer provided

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The project will provide support to work to diagnose, monitor and study viruses causing human infections.

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

What are the potential benefits that will derive from this project?

Influenza epidemics are associated with high levels of illness and death. Diagnosis of influenza infection provides a public health benefit by providing information on the number of virus infections in the population. This data can also be used to inform how well matched the vaccine is to the virus infecting the population and also how well the vaccine is performing in protecting the population.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Avian eggs
1750 eggs
Five years project duration



Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The likely severity is Mild. Expected adverse effects are from microbial contamination during the procedures. This is minimised by ensuring sterility of equipment, careful monitoring of the procedures, quality control and staff training. The embryos are terminated at the end of the procedure and before hatching.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Some viruses will only grow in embryonated eggs. Some diagnostic tests require high concentrations or volumes of virus which can only be achieved by growth in embryonated eggs.

The majority of influenza vaccine viruses are produced in eggs. Diagnostic testing associated with the influenza vaccine must use egg grown viruses to preserve the characteristics of the vaccine virus.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Wherever possible, mammalian tissue culture cells are used instead of embryonated eggs. When eggs are used the number of eggs is carefully selected according to recognised protocols to ensure that the aim of the experiment is achieved without unnecessary wastage. The number of eggs to be used is determined by the use of standard protocols to ensure the quality of the scientific outputs of the work.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Use of eggs is restricted to purposes where they provide a significant benefit which cannot be achieved by the use of mammalian tissue culture cells.

Training and sharing of protocols with other laboratories ensures that methods are updated and refined to achieve the best result.

Careful husbandry is practised to ensure minimum wastage of eggs.



48. Environmental effects on ageing

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
zebra finch <i>Taenopygia guttata</i>	neonate, juvenile, adult, aged, 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 is the aim of this project?

The aim of this project is to examine how environmental conditions influence the process of ageing in later life, using birds as a model. The project will focus on the effects of exposure to different environments at different life stages, and on the intergenerational effects of parental age on offspring longevity.

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

Why is it important to undertake this work?

It is important to understand how environmental conditions, in both early life and in adulthood, influence the pattern of age related deterioration in later life. Exposure to environments that increase stress hormone levels is known to reduce longevity but how this comes about is still relatively poorly understood.

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

We expect to generate new knowledge related to the biology of ageing and how the pattern and processes are influenced by environmental conditions at different life stages. This knowledge will be communicated via scientific publications and, where appropriate, articles in popular science media.



What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Exposures to stressful environmental circumstances, and or episodes of poor nutrition, occur in both wild and captive animals, and of course also in humans. Understanding the nature of these effects, their fitness consequences, and the routes by which they occur is important both in understanding how organisms cope with environmental change, and in potentially providing routes whereby such effects can be prevented or alleviated. Circadian rhythm disruption, for example by shift work, international travel or exposure to artificial light at night, has been shown to have adverse health consequences including accelerated ageing, but the molecular processes underlying this are poorly understood. The effect of parental age on offspring longevity is an important topic, and understanding the routes whereby parental age can reduce offspring longevity is important for both animal breeding, understanding the evolution of reproductive schedules, aspects of mate choice, and human reproduction.

This project will advance our understanding of the mechanisms that link environmental conditions in early and later life to long term health. Therefore it is important that we follow through outcomes for individuals over differing time scales and at different life stages. This is beneficial both in terms of advancing the science of ageing and development, and understanding the long term effects of environmental and growth conditions, and stress exposure. This will be beneficial to both humans and other animals. Humans and many other animals can experience early life adversity due to food shortages, inclement weather, poor physical environments, or being a subordinate. The results of this early life experience can persist long after the conditions have been ameliorated, and in some cases effects may be life long and reduce lifespan. Repeated circadian disruption can also cause long term effects. This project aims to understand the processes whereby these long term effects can occur, and may assist in the development of mitigating treatments.

How will you maximise the outputs of your work?

We will do this by appropriate dissemination of the resulting knowledge, by training young scientists in the techniques in which we have expertise and in educating students

Species and numbers of animals expected to be used

- Other birds: No answer provided
- Domestic fowl: 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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mostly, our studies are based on mild environmental manipulations and the taking of very small blood samples, typically no more than six times in the birds' lifetimes, to measure biochemical and molecular parameters. We sometimes also elevate natural hormones, but



only within the natural range, and use non-toxic markers. Typically this is done by feeding the substances to the animals, rarely where necessary by injection.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The impacts relate to responses to environmental conditions. Stress sensitivity and metabolic rate may be increased. Increased rate of growth may occur. We expect that parental age at reproduction will increase the ageing rate of offspring, and that this will be evident in the parameters that we will be measuring. We expect this to increase the rate of ageing. Lifespan may be reduced. We do not anticipate the animals experiencing any pain, weight loss or tumours. With respect to the changes in light exposure, there may be changes in activity patterns. These behavioural changes will not persist beyond the experiment itself.

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

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

Expected severities are mild for the majority (90%) of animals. For some old animals in captivity (10%) it is possible that the natural ageing process may be considered moderate.

What will happen to the animals at the end of the study?

- Kept alive
- Set free
- Used in other projects

Replacement

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

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

We are studying the effects of environmental conditions on fitness traits (longevity and reproductive performance as appropriate) and this needs to involve individual animals. We need measures of the physiological state of the animals, which is generally done from small blood samples. We also, with captive animals, need to control the environmental conditions in order to understand the processes whereby the environment influences ageing parameters. The environmental manipulations that we use are always such as to be as similar as possible to what the animals experience in the wild.

What was your strategy for searching for non-animal alternatives?

None. Not applicable.

Why were they not suitable?



We need whole organism measures of performance, and thus measures taken from cell culture are not appropriate. Our regulated procedures enable us to obtain biological samples in a minimally invasive manner, and also to control the environmental conditions to avoid confounding effects.

Reduction

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

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

Based on projected experiments over the next five years. Numbers given are maximal.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The number is based on the maximum total number to be used in the overall project. There are different components to the project, and I have endeavoured to use the minimum number likely to be required for robust experiments with adequate control groups. Where possible this is based on previous studies in which we have experience of the level of individual variability and likely effect sizes.

What other measures apart from good experimental design will you use to minimise numbers?

We breed most of our zebra finches in house in large aviaries and do not breed more than the number we require. We obtain quail from registered suppliers. Wild birds are caught at breeding, feeding and roosting sites as appropriate. We have conducted our own pilot studies in the past, or already have relevant experience of all of the protocols that we will use, or have based these on published research by other colleagues. Since we measure a number of parameters in different tissues, we make maximum use of any tissue sampling that we undertake.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We use birds because they are most suitable for our projects. We use minimally invasive procedures, mostly involving taking very small blood samples by 'pinprick'. We measure naturally occurring hormones and molecular processes. Any manipulations that we undertake are designed to mimic natural conditions. Where we sample wild birds, this



generally involves single blood or feather samples and the birds are immediately released at the capture site.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

These are not appropriate for our studies of long term effects. We use birds because they are easy to sample in the wild, and we can use species that are easy to keep in captivity. Birds are of great interest in ageing studies because of their considerable longevity (on average lifespans are 3 times those of mammals of a similar size).

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We are continually monitoring our welfare standards and implementing environmental enrichment. As well as the moral issue, we want our animals to behave as naturally as possible if they are in captivity.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

For our captive species, we use the guidelines produced by the Universities Federation for Animal Welfare. For wild birds, we follow the guidelines of the British Trust for Ornithology for the safe capture and handling of birds. The PI and other colleagues have contributed to outlining best practice for the species that we use.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

From information provided by the REDACTED, the NVS, our Biological Services staff, colleagues in other institutions and by general reading.

Explain the choice of species and the related life stages

We are studying birds because we can relate our laboratory studies to conditions in the wild, birds are recognised as particularly interesting in the context of ageing because of their long lives and repeated breeding. Their egg laying reproductive mode makes it possible to obtain early life stages without invasive procedures being used on the mothers.





49. Epigenetic and Transcriptional Regulation of Gene Expression in Neurons

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

This project will explore how gene expression is regulated in the brain during normal development and in disease.

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

Why is it important to undertake this work?

Understanding the molecular mechanism that underlies the proper wiring of the mammalian brain represents an essential step to tackle increasingly common neurodegenerative disorders, including Alzheimer’s and Parkinson’s diseases. A recent study has estimated that the cost of neurodegenerative disorders will reach £112 billions per year, including indirect costs such as lost productivity and absence from work. The laboratory has a long-term interest in employing cutting-edge technology to understand the basic mechanism that regulate gene expression in developing and adult neurons in health and disease. The laboratory has already provided the scientific community with several potential druggable targets that may be further developed into new therapeutic approaches to cure various forms of neurodegeneration.



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

The discoveries made in the laboratory in the next five years will shed new light on the basic mechanisms of brain functions and how they can be exploited to develop new therapeutic strategies to cure neurodegenerative disorders.

The impact of the findings is potentially very high.

The work proposed will characterise new basic mechanisms that control neuronal development and functions.

A wide array of innovative methodology and cutting-edge techniques will be used in neurons, thereby providing the neuroscience community with a number of scientific protocols that will greatly improve our ability to study brain development

Using a combination of mouse models, the functional significance of these mechanisms will be addressed in brain development and disease.

The innovative nature of this work should result in high profile publications that are likely to gain the attention of a broad scientific and lay audience. The public has a high level of interest in neuroscience and brain development, so the interest to the public is potentially high. For example, the American Society for Neuroscience conference attracts more than 50,000 scientists and lay people every year, and it is characterised by a number of talks by prominent personalities (e.g. the Dalai Lama) aimed at disseminating the importance of neuroscience research and its impact on society. Similarly, in the UK, the annual Neuroscience Conference of the British Neuroscience Association is attended by thousands of basic and clinical neuroscientists and many sessions are dedicated to the impact of our discoveries on human health and healthy brain aging.

Although, the impact on patients affected by autism, amyotrophic lateral sclerosis (ALS) and other neurodevelopment disorders and the public at risk of developing relevant disorders will be long-term and beyond the immediate scope of this proposal, the research will still provide an important step towards understanding, predict, ameliorate and prevent these important neurological disorders. The potential impact of this project for patients is therefore considerable, especially considering the scale of the medical problems, which include long-term disability and loss of productivity.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Academic and scientific community

The research will be widely disseminated, and the results will be discussed with scientists who are world-renowned experts in the relevant fields and present our research during national and international conferences (years 2-5).

The results of the research will be all published in high impact scientific journals that reach the wider scientific community as well as the lay public. Publications will be open access and all results will be provided in standard format, or as supplementary on-line appendices to our publications, immediately on publication (years 2-5).



The techniques developed in the laboratory will be highlighted and shared with collaborators and with other researchers during discussions in informal meetings and in the course of national and international conferences (years 1-4).

The work will also impact in terms of delivering and training highly skilled researchers, such as research technicians, graduate students and future research leaders in the field of neuroscience.

Economic and societal benefits

To ensure that the public will benefit from the research, a series of initiatives will be taken to maximise the potential impact of the discoveries on human health.

A significant impact on increasing public awareness and understanding of science will be achieved through press releases, when appropriate, and at open days during the lifespan of the project. In the annual open day for secondary school students, and work placement for students during the summer. This is aimed at introducing young students to state-of-the-art research and encourages them to consider a career in basic research and in academia. The laboratory normally participates in satellite workshops at conferences aimed at a lay audience that includes representatives of the industry, government, press and funding agencies (years 2-5).

The commercialisation and exploitation of scientific discoveries generated by the research described in this application will be a priority. Molecules and signalling pathways identified that may have relevance for autism or neurodevelopmental disorders will be tested in relevant disease models either in-house or via collaborations (years 4-5).

How will you maximise the outputs of your work?

As explained above, the research will be disseminated, and the results discussed with scientists who are world-renowned experts in the relevant fields (these include experts in neuroscience and the basic mechanisms of brain development) and the research findings will be presented in national and international conferences on an annual basis.

All publications will be open access and all results will be available to the lay public. Unsuccessful results will be published on open access journal, whenever they are considered to be of potential use to the scientific community and/or the lay public. All genomic data will be made public independently of their publication in mainstream journals and whenever the results are technically sound.

Species and numbers of animals expected to be used

- Mice: 8400
- Rats: 1100

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



The vast majority of animals (unless otherwise specifically justified) will be bred and subject to the procedures to determine their genotype. A small minority of pregnant animals will be subject to surgery that will be performed under anaesthesia and with the supervision of the veterinarian in the establishment. The surgery will be performed under general anaesthesia and will imply the transfer of genetic material and/or non-toxic substances into the brain of rodent embryos by using a very fine needle. This is necessary in order to perform a detailed analysis of the brain and to understand how lack of proper neuronal development may result in neurologic and psychiatric diseases in the adult, including schizophrenia and autism.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Potential relevant adverse effects are related to the experiment involving surgery. All pregnant females will be monitored carefully after surgical procedures and painkillers will be administered to reduce suffering whenever animals show signs of discomfort during recovery.

Manipulations of pups can occasionally lead to rejection and cannibalism. This event will be minimised by using a number of precautions, such as rubbing hands with cage sawdust before handling animals, avoiding overuse of surgical spirits and others. Animals will be monitored closely after being reunited to the mothers and fostered if possible if signs of cannibalism or rejection are evident.

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

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

The expected severity of adverse effects in the vast majority of animals is mild, unless otherwise specified as moderate. Animals subject to surgery for analysis of the brain in utero represent less than 5% of the animals and may have adverse effects due to the surgical procedure (infection of the surgical sutures and minor pain after surgery).

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Rodents (rat and mice) represent the best mammalian model to study the brain during development, as well as during adulthood in both normal and disease conditions. Importantly, the mouse is the only mammalian species that can be easily genetically manipulated and for this reason, it is widely used in the international research community for studies of the in vivo functions of mammalian genes. Therefore, in order for the data to have worldwide significance, the mouse is the most appropriate species to use.



Importantly, in rodents, nervous system development is for many aspects very similar to humans. Work conducted with this animal model will provide insights into the functioning of the human brain. More generally, anatomy, physiology, genetics of the mouse are similar to humans, and mice represent a very powerful system where to model human biology and disease. Currently, the most effective way of investigating the function of a given gene at the level of the whole animal is through the generation of genetically altered mice. Although it is possible to inactivate genes and proteins of key physiological processes by administration of toxins and drugs to whole animals, in many cases this approach suffers from lack of specificity and gives rise to more wide-ranging adverse effects that cannot be easily controlled. It also does not allow the detailed dissection of the complete network of proteins involved in key developmental or physiological processes, unlike the use of transgenic animals.

What was your strategy for searching for non-animal alternatives?

The laboratory will use cell lines (rat- and mouse-derived), whenever possible. Pheochromocytoma and neuroblastoma cell lines and other neural-derived cell lines will be normally used to optimize protocols and to perform experiments that require very large number of cells (some biochemical purification of proteins for example).

Why were they not suitable?

An alternative of the use of animals is to use cell lines to study neuronal development and degeneration in vitro. Although cell lines are used in the laboratory, the information gained is partial and only marginally helpful. Transformed cell lines do not resemble brain cells and often do not model the brain environment. In addition, the lack of reliable markers to isolate many different cell types that the lab is interested in studying represents a major problem in the field. Finally, the currently available techniques are not totally reliable as culture conditions may change the nature of the cell lines. Thus, it is difficult to model neuronal development and neurodegeneration in a dish and this is mainly due to the complexity of the brain. Intact tissues with their full complement of specialised cells represents the only system in which mechanisms can be fully tested and therapies accurately evaluated. In particular, this applies to the brain, for which insufficient information exists to generate accurate computer models, which can predict the complex responses of neuronal tissues.

Reduction

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

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

The number of animals used for each experiment has been calculated by using statistical calculations that take into account the statistical tests that will be used to analyse the results. All experiments will follow the guidelines indicated by UK research councils in order to ensure that the data are solid and reproducible by other laboratories.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?



Before embarking upon any in vivo experiments, hypotheses will be tested using in vitro models, including neuron-derived cell lines and primary neurons. When in vivo experiments are appropriate, small pilot studies will be carried out to estimate the variability of the experimental data so appropriate statistical analysis can be used to minimise the numbers of animals required for a validated result.

For all experiments, the smallest possible number of animals to obtain statistically significant differences between groups will be used, taking into account a certain degree of variability of what will be analysed.

What other measures apart from good experimental design will you use to minimise numbers?

To minimise animal usage, prevent the unnecessary production of animals showing adverse effect and to ensure that animal breeding is inextricably linked to research requirement, the project licence holder will:

Ensure high standards of animal care, welfare and utilise the most appropriate breeding methods.

Ensure that colony sizes are monitored and adjusted within a formal forecasting system to meet the requirements of the research programme(s). Transgenic REDACTED that are not expected to be used in the next 6 months will be archived and embryos will be frozen for future use

Ensure that breeding colonies are always kept to their minimum size so as not to over produce. Detailed breeding records will be kept enabling the selection of the most appropriate breeding stock.

Verify that before setting up or creating new transgenic lines a full search is carried out to ascertain that there is no other worldwide availability.

Ensure that all people working on this project are appropriately trained and suitably competent to enable a high success rate to be achieved and thus 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The laboratory will use wild type rats and mice carrying genetic modifications, as they are the most effective way of investigating the function of a gene in the brain. Although it is possible to inactivate genes and proteins during key physiological or developmental processes by administration of toxins and drugs, in many cases this approach suffers from



lack of specificity and gives rise to wide-ranging adverse effects that cannot be easily controlled. Moreover, unlike the use of transgenics, it does not allow the detailed dissection of the key developmental or physiological processes. All personnel in the lab has extensive experience in all the regulated procedures in this licence and will use their experience to ensure that suffering is minimal.

Procedures to be used:

Genetic modifications by crossing REDACTED – Models described in the project details section present only a mild form in the brain (delayed cortical formation, lower number of peripheral neurons). Although adverse effects are not expected, mice will be monitored daily and will be culled if they show signs of illness.

Genetic modifications by basic techniques. Potential adverse effects will be minimised by using state of the art facilities for surgical procedures available at the designated establishment and fully trained staff. Animals will be closely monitored after the procedure for any signs of illness or discomfort. Pain killers will be administered during the recovery phase to alleviate the discomfort.

Injections of substances needed to trace nerve cells may lead to infections in a small percentage of cases. If animals show any sign of infection, they will be euthanized immediately.

Adverse effects are expected to be rare and minor in extent.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Rats represent a very powerful model system to study the mammalian central and peripheral nervous system and they are extensively used for experiments aimed at testing the effects of environmental conditions on neuronal functions. Moreover, peripheral neurons, such as the sensory neurons found in the sciatic nerve, have been extensively characterised in rats, and this allows to use published data related to gene expression and sequencing, thereby reducing the number of experiments that will be required to test the hypothesis described in this proposal.

The mouse represents the most appropriate species to study in order to obtain data with high significance, as this model system is used extensively world-wide. Importantly, in rodents, brain development is for many aspects very similar to humans. Thus, work conducted with mouse model systems will provide insights into the diseases of the human nervous system. More generally, the anatomy, physiology and genetics of the mouse are similar to humans, and mice represent a very powerful system where to model human biology and disease.

Less sentient species such as the fly *Drosophila melanogaster*, the fish zebrafish, and the worm *C.*

elegans are used to study some aspects of the nervous system functions and are considered appealing as they can be genetically manipulated very easily. However, it should be noted that the brain of these species is extremely different compared to human or mouse as they contain only 135,000 neurons in *drosophila*, 10 millions in zebrafish and only 302 in *C.elegans* (as a comparison, the mouse brain has about 100 millions neurons and a similar number of non-neuronal cells, whereas the rat has more than 200 millions



neurons). Therefore, the number of connections between cells and the structure of the brain is very different in these species when compared to rodents and humans, and this is due to the distinct set of behaviours that the brain must control in non mammalian species. Importantly, our research is based on the discovery of new genes and new gene functions that may play a fundamental role in regulating the mammalian brain in health in and disease. In the past decade, extensive sequencing of most genomes has shown that many genes expressed in rodents and humans are not found in lower species. Thus it would be impossible for us to investigate for example, the mechanisms that regulate the formation of the brain and the cause of neurodegenerative and psychiatric disorders observed in the adults using species that are so genetically distant from humans.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The Biological Services at the institution run a comprehensive health-monitoring programme. Animal health and welfare records are maintained to include any adverse effects that may develop, particularly in genetically altered and spontaneous mutant strains. Signs consistently associated with a particular strain will be recorded on the respective “information sheet” in the breeding area (see above). The animals will be maintained under conditions where their health status can be protected as far as is reasonably practicable.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The project holder 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 Royal Society for the Prevention of Cruelty to Animals (RSPCA).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

All associated staff in the lab has extensive training in all the regulated procedures in this licence, and will use their experience to ensure suffering is minimal. The staff will attend all recommended courses organized by the Biological Services at the institution to maintain best practice and to keep our knowledge of animal welfare up to date with the Home Office guidelines.

Explain the choice of species and the related life stages

The laboratory uses rodents (mice and rats), as they provide an ideal model system to study the brain in both normal conditions and in disease. Rats and mice share many of the anatomical and physiological features with humans and represents faithful model systems to study nerve development, maintenance and regeneration in health and disease. The aim of my research is to study the mechanisms underlying brain development and disease and the use of rodents will allow me to meet my scientific objectives and accrue benefit.





50. Expression of cardiac mutations in Zebra Fish

Project duration

5 years 0 months

Project purpose

None selected

Key words

No answer provided

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

To determine the role of specific gene mutations found in the Egyptian population on heart function

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

What are the potential benefits that will derive from this project?

Understanding the genetic basis of heart failure to improve detection and treatment of this condition

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

We expect to use up to 25,000 fish over a 5 year period

Predicted harms

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



In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Some fish may develop impaired cardiac function, of a mild to moderate severity. At the end of the end of the experiments the fish will be euthanised.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Cardiac function can only partially be modelled using cell culture models. Expression of gene mutations in isolated cells and assessment of the function of the cells would not give a full assessment of the phenotype and function of a whole organ, Such experiments cannot substitute research in animals.

Reduction

Explain how you will assure the use of minimum numbers of animals.

We shall only use a minimum number of animals in our experiments. Breeding strategies are performed by qualified personnel and aimed to avoid unnecessary animal generation. Results will be published according to the ARRIVE Guidelines.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The zebrafish model is an excellent model organism, which due to its transparency of the embryo, its high fecundity, its rapid development and the availability of sophisticated genetic tools provides vast opportunities to model the genetic basis of cardiovascular and skeletal muscle disease. We will be using the zebrafish to introduce mutations found in the Egyptian population to ascertain the functional impact these mutations have on the heart. The phenotypes often become first apparent during embryonic development and thus suffering of the animals will be minimised due to the short period until the cardiovascular and muscle pathologies become apparent. Any fish undergoing treatments will be monitored for infection, poor healing or altered schooling behaviour and killed using a humane method to minimise suffering if necessary.





51. Fish behaviour and welfare

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

No answer provided

Animal types	Life stages
Zebra fish	embryo, neonate, juvenile, adult
Teleost fishes	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 is the aim of this project?

To understand how fish behaviour and welfare is influenced by environmental factors in both artificial (e.g. aquaculture, ornamental fish trade) and natural contexts.

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

Why is it important to undertake this work?

In order to determine the best ways to protect fish welfare under commercial settings and to protect natural populations against environmental change, there is a need to understand how changes in their environment affect fish behaviour and physiology (Objective 1 of this project). For example, changes in water quality during transport of fishes for aquaculture can lead to increased stress levels and decreased welfare. Within the natural environment, low levels of contaminants can cause subtle changes in fish behaviour that can have serious implications at the population level if they interfere with the ability of fishes to catch



prey or avoid predation. In addition, we need to understand how environmental changes affect the early life stages of fishes; small effects at these sensitive stages can have knock-on effects throughout the life cycle. It is also becoming clear that the environmental conditions experienced by parents can affect the behaviour and welfare of subsequent offspring. Thus this project aims to further our understanding of how fish behaviour and welfare is influenced by environmental factors. Traditionally, monitoring the way fishes respond to environmental change has utilised invasive or terminal sampling; therefore alongside this fundamental knowledge there is a need to develop robust non-invasive, or minimally-invasive, measures of stress in fishes (Objective 2 of this project). The benefits of this research are discussed in detail below.

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

Outputs from this project will include new information on the factors which affect physiology and behaviour of fishes, crucial for determining the best ways to protect their welfare and protecting natural populations. New information will be made available primarily through peer-reviewed publications, industry reports, scientific conferences and public presentations.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

From a welfare perspective, these outputs will be of benefit for the welfare of fishes held both within the ornamental trade and aquaculture and can be used by these industries to improve best practice. The ornamental trade represents a multi-million pound industry where millions of fishes are transported and held as pets. Ornamental 'pet' fishes (e.g. guppies, zebrafish, neon tetras) lead very different lives to fishes such as salmon and trout held in aquaculture, and yet much of our understanding of ornamental fish requirements (e.g. diet, water quality), is based on information available for large aquaculture fishes. Very little research has looked at nutrition, husbandry, stocking and transport of ornamental fishes in terms of their welfare. This project looks at ways of improving fish welfare by increasing our understanding of fish behaviour and physiology and how non-invasive methods can be applied to assess fish welfare (e.g. observing behaviour, measuring hormones released into the water and taking mucus samples). Within the food-fish aquaculture industry there is also an increasing desire to improve welfare through the use of more behavioural and non-invasive monitoring. Therefore, this project has the potential to improve fish welfare across a wide range of settings.

Understanding the interactions between behaviour and physiology in fishes is also important for conserving natural populations. In making decisions about protecting our natural environment, often shifts in fish behaviour induced by environmental change have been over-looked or deemed insignificant. Yet small, seemingly unimportant, changes in behaviour can have serious implications for a population if they limit a fish's ability to feed or to escape predation. In this context it is also important to develop non-invasive measures of fish health to allow monitoring of natural populations with minimal disruption.

How will you maximise the outputs of your work?

Our research group works closely with industry (for example with wholesalers and retailers of ornamental fishes and food fish aquaculture companies) and therefore can ensure that results are disseminated in a timely fashion, often considerably prior to publication in the peer-reviewed literature. Unsuccessful approaches are also discussed with our industrial



partners so that refinement of approaches, and consideration of 'non-significant' effects occurs.

Species and numbers of animals expected to be used

- Other fish: No answer provided
- Zebra fish: 1500

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, fishes will first be exposed to a change in environment which may be procedural as it has the potential to cause an adverse effect (e.g. provision of a diet with a lower than normal level of protein, experience of a transport stress) or non-procedural (e.g. addition of environmental enrichment).

Following this fishes will be monitored for changes in behaviour and physiology. Behavioural measurements may include: ability to perform in a maze environment (procedural), to compete/socially interact with other individuals (procedural), and other non-procedural measures including activity, feeding, interaction with enrichment. Physiological measurements could include: mucus sampling (procedural), blood sampling (procedural), respirometry and/or swimming ability (procedural) and other non-procedural measures including water-borne hormone levels, body colouration.

At the end of an experiment, fishes may be released from the act if they are not suffering, or likely to suffer, any adverse effects of regulated procedures. This will be determined by a veterinary surgeon or a competent person using criteria agreed with the named veterinary surgeon in line with standard project licence conditions.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Potential (rather than expected) adverse effects from this project could include adverse changes in behaviour (e.g. lack of feeding, loss of equilibrium/swimming ability, laboured respiration) and skin abrasions. Any fish showing signs of these adverse changes will be immediately removed from the experiment and euthanised by a Schedule 1 method if recovery is unlikely, thus the duration of these effects would be minimal.

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

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

With the exception of fishes exposed to aquatic toxicants (5%) the expected severity of procedures is mild. The work involves exposing fishes to mild stresses that they would



naturally encounter in the wild or in an artificial environment (e.g. in aquaculture or as pets). For those fishes exposed to aquatic toxicants, very low levels of contaminants are used to look for subtle changes in behaviour and physiology, therefore it is unlikely that adverse effects will be seen. However, due to the nature of these experiments they are classified as moderate. It is anticipated that the majority (95%) of fishes from this project that have only been exposed to mild stresses will be released from the act either to stock or re-homed as companion animals where possible.

What will happen to the animals at the end of the study?

- Kept alive
- Rehomed

Replacement

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

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

Currently there are no *in vitro* or computer-based alternatives for this type of research. We have used the www.frame.org.uk website and associated links to look for alternatives and can find none documented but will continue to monitor both this website and the scientific literature to look for alternatives. Where possible we use non-invasive measures (e.g. behavioural observations of fishes during commercial practice) to address our objectives.

What was your strategy for searching for non-animal alternatives?

There are no *in vitro* or computer-based alternatives for this type of research.

Why were they not suitable?

There are no *in vitro* or computer-based alternatives for this type of 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?

For all experiments associated with this project, experimental design is based on statistical planning. Careful consideration of existing literature, including our own unpublished and published data is used to inform experimental design. Tank replication has also been considered as there may be variability caused by different social groups within tanks. This allows us to use the minimum number of fishes while ensuring statistical robustness.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?



Consultation with statisticians (in-house and/or industry based) occurs both during experimental design and of the final experimental design prior to the start of experiments. For research involving industrial partners, additional statistician input to data analysis approaches allows us to continuously refine our experimental designs and to minimise the number of fishes being used.

What other measures apart from good experimental design will you use to minimise numbers?

For work with embryos, many measurements can be made prior to hatch with only ~25% of embryos used being raised to first feeding and becoming licenced fishes. Therefore, some partial replacement occurs. Additionally, we continue to develop non-invasive methods of measuring fish welfare, and to work with industry to monitor and refine fish welfare under existing commercial practice, thus reducing the need for experimental research on fishes.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The species used in this project will be typical teleost fishes (mainly freshwater) that are either considered to be model species for fish behaviour and physiology (e.g. zebrafish and trout) or are important commercial species (both for food-fish aquaculture and the ornamental trade). These will include species from the families Goodeidae and Cichlidae among others.

For each objective and experiment, appropriate fish species will be chosen based on sound scientific reasoning. Many of the research questions posed in this proposal are related to ornamental fish species and in considering social behaviours and responses to husbandry stressors it will be necessary to consider species with a range of life histories, such as live-bearers and egg-laying species.

In the protocols, different methods of carrying out a procedure are given where different species may require different procedures. Where a choice of methods exists, the mildest method to achieve the stated objectives will be chosen.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Our previous work has refined the use of techniques to assess the impact of environmental change on very early life stages, thus reducing the need to raise embryos beyond first feeding. We will continue to use these methods where they can appropriately address the objectives. However, to understand impacts of husbandry and environmental



stressors on fish behaviour, it is not possible to use species that have been terminally anaesthetised or are less sentient.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Welfare costs associated with this project are expected to be mild. Fish health and welfare will be monitored on a daily basis by experienced staff (including the NACWO) including feeding behaviour, swimming behaviour and overall condition of fishes. Water quality parameters are checked on a daily basis.

Protocols 2 and 3 (of 3) are mild and chosen for their sensitivity in detecting differences in physiology and behaviour. Protocol 1 is classed as moderate due to the potential exposure to aquatic toxicants (N.B. this is an optional component of this protocol) but only mild effects of the toxicant on behaviour and physiology are expected due to the aim of the objectives being to understand the effects of sub-lethal doses. There is an extensive body of literature documenting the effects of aquatic contaminants on fishes, particularly with regard to lethal and acutely toxic concentrations. Here the effects of chronic exposure (ideally at the lowest observable effect concentrations) are of interest and suitable concentrations will be calculated by carefully scaling down concentrations known to be acutely toxic. For all protocols, where a choice of methods exists, the mildest method to achieve the stated objectives will be chosen.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We use a variety of practice guidance to ensure refinement of experimental techniques. These include, but are not limited to:

Guidelines for the treatment of animals in behavioural research and teaching (ASAB, 2012, 2018).

Ethical justification for the use and treatment of fishes in research (Journal of Fish Biology, 2006; Metcalfe & Craig, 2011).

Guidelines for the use of fishes in research (AFS, 2014).

ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines (Kilkenny et al., 2010).

PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) Guidelines (Smith et al., 2018).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We regularly consider updates on the NC3Rs website and receive information through AWERB meetings and email communications. Both PI and students have previously and will continue to attend LASA, RSPCA and NC3R events, including those specifically focused on fish research. Where advances in the 3Rs, particularly with regards to refinement of working with fishes occur, we look to implement these within our work.



Explain the choice of species and the related life stages

The fish species used in this project will be mainly freshwater fish species that are either considered to be model species for fish behaviour and physiology (e.g. zebrafish and trout) or are important commercial fish species (both for food-fish aquaculture and the ornamental fish trade). It is important to understand the response of all life stages of fishes to husbandry practices and environmental change; indeed we know that husbandry conditions of female fishes can affect subsequent behaviour of their offspring. Therefore, all life stages will be considered.





52. Gene-Environment Interactions

Project duration

1 years 5 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

This program of work aims to better understand the consequences of exposure to environmental pollution on living organisms. We will use a panel of reporter mice to study how environmental pollution activates different toxicity pathways and causes exacerbation of disease.

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

Why is it important to undertake this work?

Humans are constantly exposed to a complex mixture of deleterious environmental agents including pollutants in air, water and foods; and on occupational and household settings. Epidemiology studies have linked exposures in adulthood and during development to the rising incidence of non-communicable diseases and behavioural deficits. The WHO



estimates that 23% of global deaths are attributable to environmental factors such as air (e.g. particulate matter, nitric oxide), water (e.g. arsenic, heavy metals) and soil contaminants (e.g. pesticides). Despite these statistics, in most cases we do not understand at a mechanistic level how complex mixtures of pollutants disrupt cellular and physiological homeostasis to exacerbate disease pathogenesis.

Whereas significant progress has been made in the identification of hazardous environmental exposures and their consequences on cell homeostasis, the majority of these conclusions are based on *ex vivo* studies. Proposed mechanisms include oxidative- and DNA-damage, and Aryl hydrocarbon receptor (AhR) interactions. However, the majority of the current understanding does not take into account the complexities of cell-cell interactions and whole animal physiology.

Our program of work will provide *in vivo* mechanistic insights on gene environment interactions. We will measure the activation of adaptive stress responses by environmental pollutants using a panel of reporter mice of oxidative- and DNA-damage and AhR-activation. These studies will include the consequences of *in utero* and trans-lactational exposure. In the future, we will use this information to interrogate how individual genetic variability influence susceptibility to environmental exposure. Finally, the identification of pathways activated by environmental exposure can be used to inform subsequent epidemiological studies and can provide biomarkers to test in human studies.

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

The immediate outputs of this project will be scientific publications in high quality, peer-reviewed, high impact journals appropriate to the field of research, communication to relevant national & international scientific conferences and press releases disseminating the research findings.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The public health consequences of environmental contaminants are a major global health priority. Human exposure to a complex mixture of deleterious environmental agents including pollutants in air, water, and foods and occupational and household chemicals has been linked to the rising incidence of non-communicable diseases and behavioural deficits in adulthood in both wealthy and developing countries. Indeed, WHO estimates that 23% of global deaths (36% amongst children under 14), are attributable to environmental factors. In the short term, the project will benefit the scientific community to understand the mechanisms of toxicity by environmental exposure. The reporter models used will allow fundamental advances on this research, such as a better understanding of cellular sensitivity to pollution *in vivo* and avoidance of technical problems associated to current methodology (i.e. lack of cell-specific biomarkers). In the medium term, this information will be used to build up more robust models to interrogate how pollution is linked to initiation and exacerbation of human diseases. In the long term, the project will benefit the society through an increased understanding of how environmental pollutants lead to disease and, in the longer run, through contributing to improvements to the quality of the environments in which people work and live.

How will you maximise the outputs of your work?



The mounting global concern surrounding the public health consequences of environmental contaminants will ensure the impact of this work. The knowledge and skills acquired will be deployed to understand the consequences of emerging environmental toxicology problems, such as household/indoor pollution, tear and wear of tyres and toxicity of new materials (e.g. microplastics). To maximise the output of this work, we will ensure the continuity of our research lines by extending our current national and international collaborations with leading figures in the field of toxicology .

The stress response models can be applied to a number of toxicological problems. Therefore, we will seek new collaborations with toxicology groups/centres where environmental research is a priority.

The work described in this project will be disseminated through publications in peer review journals and by presentations at scientific meetings.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will be treated with agent(s) associated with environmental pollution (diesel emissions, singularly or in a mixture; naturally occurring pollutants; indoor pollutants, singularly or in a mixture), with all substances and procedures shall be pre-approved locally by the NVS. A typical scenario may be daily po doses of a pollutant for a maximum of 5 consecutive days. Occasionally, antioxidants can be co-administered in the diet or drinking water. After the exposure period, mice may undergo optical imaging before sacrificed by exsanguination and/or tissue perfusion under terminal anaesthesia [AC(G)] or by a Schedule 1 method.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The main type of impact on animals during this project will be a transient discomfort during the administration of the substances. In rare occasions, animals may develop pain and weight loss. Animals experiencing this type of effects will not be included in our studies and will be killed by a Schedule 1 procedure after consultation with NVS or NACWO as appropriate.

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

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



Mice in protocol 2 will be expected to experience a mild severity. For animals with treatment in drinking water or diet (15%) the severity will be below threshold. In rare occasions, less than 2% cases, animals may experience a moderate effect. For instance, animals may refuse the modified water or food as a consequence of dissolved compounds making it less palatable, with a concomitant weight loss or dehydration.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 variety of environmental exposure whose links with stress responses we wish to explore affect multiple organs and can only be investigated in an intact organism. Although in an ideal world, some of these studies would be performed *ex vivo* using human cell lines, these lines are invariably cancerous in origin and the process of cell transformation alters how cells produce, use and adapt to environmental pollutants; again, this makes this approach unacceptable for our purposes.

What was your strategy for searching for non-animal alternatives?

Potential work is developing technologies for engineering stress-reporters, similar to those described herein, into induced-pluripotent stem (iPSC) cells. In future, this will allow us to replace mice in some experiments with iPSC cells or immune cells derived from exposed individuals. But, for now, mice remain irreplaceable and the organism of choice for our work.

Why were they not suitable?

To understand the consequences of environmental exposure and their role in disease aetiology, there is a need to work at the organism level. iPSC cells will not reflect the complex cell-cell interactions of the whole organisms. Moreover, pollutants often require metabolic activation to reactive species in order to exert their damaging effects. This metabolic activation is often cell-dependent and iPSC cells do not express the required set of metabolic enzymes. Metabolites can also exert their effects in tissues distant from their metabolic activation site. Therefore, for a comprehensive study of pollution toxicity, a whole organism is required at the moment.

Reduction

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



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

The number of animals required for this project has been calculated on the basis of previous experience with these lines. We have also estimated the number of pollutants and complex mixtures available for our research and the experiments required to understand their toxicity mechanisms.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Experimental design – based on experience and literature review - will be kept as simple as practically possible in order to maximise the information obtained from the minimum number of animals. Advice will be sought as necessary from statistical sources, locally or online, concerning the minimum number of experimental animals required to allow a sufficiently powerful statistical analysis. Experience to date suggests that three mice per experimental group will usually be acceptable. This is the smallest number of animals that could be used consistent with good statistical practice.

What other measures apart from good experimental design will you use to minimise numbers?

The animals required for our experiments are heterozygous for the reporter allele. To ensure an efficient breeding, we will cross wild type animals with homozygous reporters, therefore all the offspring will be amenable for experiments.

As aforementioned, we conducted a comprehensive characterization of pollutants ex vivo before proposing their use in an in vivo study. This approach ensures the animals used for our research are kept to a minimum.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use mouse models which are reporters for the activation of the NRF2 pathway (oxidative stress; HOTT, NRF2-KO), p53 pathway (DNA damage; p21), AhR pathway (xenobiotics receptor; CYP1a1_KI_Cre/multireporter) and AP-1 pathway (early stress response; Pyroz). The presence of the reporter has no deleterious effects on the animals. The severity of the methods to be used has been classified as mild.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The regulation of stress pathways can differ between different species. Mice represent a closer step to understand the consequences of environmental exposure on human health.



What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals that will enter into a study are acclimatized to handling. When animals are in a study plan, we will increase the monitoring of adverse effects.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

There are multiple online resources which are updated with best practice guidance for experiments, e.g. LASA website or the NC3Rs website.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I will regularly visit the NC3Rs website and others recommended by the local named information officer.

Explain the choice of species and the related life stages

Of the model organisms commonly accepted as surrogates for humans, the mouse is the closest in evolutionary terms to humans. Simpler, more socially-acceptable alternatives, such as the worm species *C. elegans*, have well-documented differences with humans in how they handle oxidative stress and this makes them unacceptable for this project.





53. Generation of antibodies in pigs against the SPIKE protein of Wuhan 2019 Corona Virus (SARS-CoV-2)

Project duration

1 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Pigs	adult, juvenile

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

To generate polyclonal and monoclonal antibodies in pigs to the SARS-CoV-2 spike protein to both enable reagents to be available for treatment and diagnosis and also to be used to map the surface of the virus against which antibodies are produced. Another aim is to assess the effect of route of administration of the human experimental vaccines on the subsequent immune response and kinetics.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 there are currently little to no reagents readily available to help develop diagnostic tools, in particular antibodies which are used in a variety of diagnostic tests for other infectious diseases. Producing monoclonal antibodies also will allow the portions of the Coronavirus surface which elicit an immune response to be mapped and compared with other animal models in order to select an appropriate



model for immunogenicity. The immune responses to experimental human COVID-19 vaccines are essential to define, and this study will use the pig model to determine the detailed immunogenicity of human experimental vaccines, including the effects of different routes of vaccination on the subsequent immune responses.

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

At the end of this project it is predicted that immunological materials (polyclonal and monoclonal antibodies) will be produced against the Spike protein of SARS-CoV-2. These will help investigate and diagnose the ongoing SARS-CoV-2 epidemic to which there is a paucity of materials available at present. Immune responses of experimental human COVID-19 vaccines administered by differing routes and schedules of immunization will be determined which may help identify the optimal regimens and in doing so allow a more efficient use of the vaccines, thereby increasing the number of potential doses available.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The antibodies produced in this study (both polyclonal and monoclonal) will be made available to our collaborators in the short term. Novel diagnostic tests, as well as mapping the parts of the SARS-CoV-2 to which the immune system creates antibodies (epitopes) will be achievable in the medium term. In the long term, it is envisaged that the reagents created from this work, and associated derived assays, will aid in the creation of countermeasures (such as a vaccine and therapeutic antibodies) to SARS-CoV-2 where the ultimate benefit will be to patients and the global at-risk population as a whole.

Knowing how the pig immune system behaves when the animal is vaccinated with the human COVID-19 experimental vaccines will allow the vaccination approaches in humans to be optimised and understood with much more clarity, especially the potential induction of vaccine induced enhanced disease.

How will you maximise the outputs of your work?

Collaboration with structural biologists in REDACTED will allow the epitopes of the Spike protein to be mapped. This will be helpful in designing or testing novel diagnostic reagents as well as disease countermeasures.

Collaboration with teams having access to human and mouse monoclonal antibodies (mAbs). Compare the pig mAbs with human mAbs to establish if humans or pigs are better in making neutralizing mAbs. If pigs and humans make a similar repertoire of mAbs pigs could be a useful model to test the immunogenicity of SARS-CoV-2 vaccines.

The reagents created in this study will be available to collaborators in the global effort to investigate the immune response, as well as creation of countermeasures to SARS-CoV-2.

Close collaboration with REDACTED, and ongoing sharing of results will allow frictionless information exchange regarding the immunogenicity of the experimental human COVID-19 vaccine.



Species and numbers of animals expected to be used

- Pigs: 66

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, Animals used in this project will injected into the muscle or skin, or administered material into the nose or lungs with SARS-CoV-2 proteins or vectors expressing the proteins to induce an immune response. This will typically be conducted twice. Blood samples and swabs will also be taken to characterize the immune response and to isolate immune cells for further analysis. This will typically be done weekly. After around 56 days animals will then be culled humanely. Some pigs may also require sedation to enable accurate administration of vaccine. After culling, various immune tissues will be collected to further collect immune cells which will then be used for further analysis.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

There are no adverse effects expected during this project other than the brief discomfort associated with an injection (of the antigen) and blood sampling. The substances inoculated into the pig are not expected to cause any adverse effects themselves.

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

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

The maximum severity expected in all pigs is mild, and 100% of pigs used in this study will experience it.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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



Due to the complex nature of the immune system, it is not currently possible to generate antibodies (or to accurately predict them) to a novel protein without inoculating it into an entire animal.

What was your strategy for searching for non-animal alternatives?

It is not possible to achieve this aim without using animals as above. Cell culture based systems will be used to generate the SARS-CoV-2 proteins.

Why were they not suitable?

No replacements options are available to replace the whole animal at this time as an entire organism, including the immune system, need to be present.

Reduction

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

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

The first study is a pilot study to generate the antibodies to the SARS-CoV-2 virus spike protein. Based on previous work, 3 pigs are sufficient to produce a broad set of monoclonal and polyclonal antibodies. As we will be using two immunogens, we will be using 6 pigs. The subsequent studies will involve development and use of a pig model to examine the immune responses to human experimental COVID19 vaccines. These studies will be able to investigate the optimal dose, regimen, and route of vaccination. Numbers of animals per groups, and number of groups will be determined based on previous data collected by working with a statistician.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Review of the previous studies generating monoclonal antibodies have confirmed that 3 pigs are required. Using statistical approaches it was determined that 6 pigs will be required to determine the effects of route of vaccination on the responses.

What other measures apart from good experimental design will you use to minimise numbers?

The first is a pilot study itself, and the numbers have already been optimised. The second is an immunogenicity study and responses based on recently acquired data in mice.

Refinement

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



Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will be using pigs because we have previously shown that antibodies generated by pigs recognise similar epitopes to human antibodies, in contrast to antibodies raised in mice, when investigating influenza viruses. The method of isolating and creating antibody secreting cells in pigs has also been validated previously and so will maximise the chances of generating results in this model. The methods used are the minimal number and consist of a vaccination with a needle (and instillation into the nostrils) which is the least pain needed to generate antibodies.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Pigs have been used in this study as there is evidence that the antibody response of pigs to influenza virus proteins is similar to that of humans, and these influenza proteins are similar to the coronavirus spike protein. Furthermore, there is a concurrent mouse pilot study which will compare the antibody responses of the mouse to the pig, to elucidate differential recognition of the SARS-CoV-2 spike protein. Pig monoclonal antibodies are antigenically different from mouse antibodies, and therefore there is less chance of cross reactivity if they are both included in certain diagnostic and research assays.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals will be housed together with bedding and other items of enrichment. Highly trained animal technicians will monitor these animals throughout the day, ensuring they are comfortable and to maximise their welfare status. We have 24/7 CCTV surveillance which can be used to monitor the animals behaviour over time.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Adherence to the ARRIVE guidelines for reporting these studies, as well as reference to the FELASA guidelines for pig health monitoring to help ensure the most robust health assurance for animals used in this study. FELASA guidelines for administration of substances has been used to limit the maximum volumes for each of the routes.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Through continued CPD and frequent review of the CAAT (Center for Alternatives to Animal Testing) I will keep informed about advances in the 3Rs. Included in CPD will be annual attendance at national lab animal science conferences.

Explain the choice of species and the related life stages

Pigs are being used in this study as they have previously been demonstrated to generate an immune response in line with the human responses to influenza antigens (which are similar in configuration to SARS-CoV-2 spike proteins). Pigs are also naturally infected



with coronaviruses which may further aid to them being a future model, and therefore another benefit of using them as a model to create reagents. Also, the large size of the pig compared to rodents allows B-cells in the blood to be characterized and isolated, in addition to the large amounts present in the secondary lymphoid tissues. The size and anatomical similarities of pigs make them ideal for assessing the effect that the route of vaccination has on the immune responses.





54. Homeostasis and disease in multilayered epithelia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Some tissues, called multilayered epithelia, act as a protective interface between our bodies and our environment, for example in the skin and mouth. The aim of this project is to understand how they function in health and disease, so that we can develop new ways to treat diseases such as cancer and skin 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?

Multilayered epithelia, such as the epidermis, cervix, oesophagus and oral mucosa, act as a protective interface between our bodies and our environment. In part because of their exposure to environmental carcinogens, and in part because they turn over rapidly, they are frequent targets of neoplastic conversion. Such cancers are a major human health problem. Epidermal basal and squamous cell carcinomas (SCCs) are the most common type of cancer in the UK and although they can normally be cured by surgery SCCs can



metastasise (spread) if left untreated. SCCs of the head and neck (HNSCC) are less common than epidermal SCCs but have relatively high mortality: 11,900 people in the UK are diagnosed with HNSCC every year and there are approximately 4,000 deaths. The 5-year survival rate is 28-67%. HNSCC represent an enormous burden to a patient, since surgery and adjuvant therapies are mutilating and have a devastating effect on normal oral function as well as general health and mental wellbeing. New treatments are required that are more effective and less damaging to the patient.

In addition to their importance in cancer, multilayered epithelia are deregulated in a number of common inflammatory conditions such as psoriasis and eczema. All such benign disorders are incompletely understood and are believed to represent an attempt by inflammatory cells to protect the tissue from impaired barrier function.

Multilayered epithelia are maintained throughout adult life by proliferation of a subpopulation of cells known as stem cells. These cells not only self-renew, but also generate all the differentiated cell types of the epithelium such as hair follicles and sweat glands of the skin. Stem cells give rise to tumours when they undergo changes to their DNA and undergo uncontrolled growth. However, it is still unclear what determines tumour type and, in addition, there is evidence that differentiated epidermal cells can influence tumour development in a positive or negative fashion.

In order to study the epithelial stem cell compartment and its contribution to normal differentiation and disease, four important experimental tools are available. First, it is possible to culture cells from normal human and mouse epidermis (and other multilayered epithelia) and to reconstitute the tissue in culture. Secondly, histological sections of normal and diseased tissue can be examined and used to evaluate the significance of the in vitro observations. Third, it is possible to make transgenic and knockout mice in which gene expression is altered either in the stem cells or differentiating cells. Fourth, small pieces of human or mouse tissue, cells isolated from tissue or cultured cells can be grafted into suitable recipient mice to evaluate their growth and differentiation potential. Our observations using the first two approaches inform our decisions to carry out experiments with mice. We also access electronic databases of information to make predictions about how the properties of multilayered epithelia are controlled. Wherever possible my laboratory carries out experiments on cultured cells and human material. However, tissues formed from multilayered epithelia are too complex to be fully recreated in a culture dish and for this reason it is essential to carry out some experiments on mice.

In particular the formation of a tumour depends on different cell types such as blood vessel cells and immune cells and it is possible to control tumour growth by targeting those cells as well as the cells of multi-layered epithelia – these types of interactions are so complex that they need to be analysed in a living mouse.

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

The major outputs are new information about tissue homeostasis and disease; REDACTED; wide spread dissemination of knowledge through publications and presentations at seminars and conferences; and proof of concept research for clinical applications. A further output is to maximise the value of the mice that we study by sharing tissue with other researchers. This is because the genes we study in tissues with multilayered epithelia are often expressed in other tissues. By making those tissues available to other researchers, mice that are killed for this project can foster research on other disease relevant tissues, including breast and intestine.



What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

This work is expected to provide novel information about the properties of stem cells of multilayered epithelia, how they communicate with other cell types in the same tissue, the pathways that regulate their differentiation, and the changes that result in benign hyperproliferative disorders and in tumour formation. Tumours of the epidermis, called basal cell carcinomas and squamous cell carcinomas, are the most common tumours in humans. Tumours of other multi-layered epithelia, such as the oral cavity, cervix and oesophagus, are less common but have significant mortality. Benign skin conditions are also a major health problem, with 2% of the world's population estimated to suffer from psoriasis. New insights into the underlying mechanisms of these diseases will be generated continuously throughout the project. Clinical applications, such as new drugs, will occur over a longer time frame of up to 10 years from completion of the project. Nevertheless, the pace of translation is accelerating, and discoveries made in my lab since 2013 have already attracted interest in new treatments for scarring and in stratifying combinations of drugs for head and neck cancer on the basis of the underlying genetic lesions. The scientific community will benefit throughout the project from sharing data, protocols and reagents, including any surplus tissue. Staff working on the project will gain training in the use of animals and as a result will have enhanced career opportunities; for example, over 50% of researchers leaving my lab go on to independent academic careers.

How will you maximise the outputs of your work?

In recent years we have increasingly used experimental data generated from mouse models as the basis of collaborations with mathematicians and computational biologists. These collaborations result in a reduction in the number of mice we use, because the computational models make firm predictions about further in vivo experiments. My lab is committed to dissemination of new knowledge through presentations at conferences and conventional peer-reviewed publications. In addition we share unpublished data via pre-print servers such as BioRxiv and the protocol sharing website protocols.io. Through our commitment to maximising the output of our experiments we intend to publish work that yields negative results or is confirmatory via routes such as BioRxiv and ScienceMatters.

Species and numbers of animals expected to be used

- Mice: 45,500

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mice may be subject to procedures such as ear snipping and blood sampling for genotyping and to monitor the immune status of the animal. Mice will typically be subject to topical applications of agents such as Tamoxifen, or receive injections of labelling agents. They may be subject to wounding or UV irradiation to induce skin damage and may be transplanted with cells or tissue fragments - normal or cancerous - either in suspension or



in chambers. They may be treated with chemical carcinogens, either in the drinking water or by skin painting in order to stimulate the formation of tumours. They may be treated with potential anti-cancer drugs and their immune system may be altered, for example as a means of potentially slowing cancer progression. They may be anaesthetised and subjected to in vivo imaging. Most experimental observations are complete within 1 week to 3 months.

In this project mice may develop cancers of the skin or oral cavity. Cancer studies typically last 7 to 12 months. Typically tumour development can be evaluated by visual inspection and tumour metastasis is, in our experience very rare (fewer than 5% of tumour bearing animals). However, when tumours form in the oesophagus or forestomach – as a result of 4NQO carcinogenesis - they are not visible and therefore careful monitoring of weight is important, weight loss being indicative of tumour formation.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Expected impacts are the development of benign skin disease and skin and oral tumours. Provided that the mice are showing no other signs of ill health they may experience these effects for several months.

We do not expect any of the following clinical signs: intermittent vocalisation; pallor of eyes, nose, ears and food pads.

Any weight loss is expected to be transient (up to 3 days), except in the 4NQO protocol, when there may be slow weight loss for the final weeks.

Other phenotypes indicative of a mouse that is in pain or experiencing stress - hunched posture, reduction in food and water consumption, a disheveled coat, subdued behaviour and abnormal breathing - are expected to be rare 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 species)?

In protocol 1 the severity limit is 'mild' and it is expected that over 80% of mice have no phenotype at all.

In protocols 2-6 the severity limit is 'moderate' and it is expected that 80% of mice will experience this severity. Moderate phenotypes will include formation of tumours of the skin or oral cavity.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 behaviour of keratinocytes, the cells that form multilayered epithelia, in health and disease is profoundly influenced by communication with other cell types, such as skin fibroblasts and bone marrow derived cells. These interactions cannot be adequately recreated in culture.

Furthermore, cancers develop over a period of months and it is not possible to maintain keratinocytes in culture for more than 21 days without subculturing them. We have also found that the way cells behave in culture does not fully recapitulate the way they behave in the body - for example fibroblasts proliferate rapidly in culture but only rarely in undamaged adult skin. Furthermore, the effectiveness of anti-cancer and anti-inflammatory drugs depends not only on the drug target but also on pharmacokinetics and -dynamics, parameters that can only be assessed in vivo.

What was your strategy for searching for non-animal alternatives?

Keratinocytes (the cells of multi-layered epithelia) can be grown in culture and induced to differentiate into interfollicular epidermis and sebaceous gland cells. Histological sections of human epidermis and other multilayered epithelia are available for analysis. Data sets of gene expression and genomics are available and it is possible to run computer simulations of aspects of tissue behaviour, including wound healing.

Why were they not suitable?

Analysis of different datasets and computer simulations is very effective at hypothesis generation, but validation requires mechanistic experiments that can only be carried out in cell culture or in mice. As described above, cell culture cannot mimic the full complexity and extended time course of the interactions between different cell types that influence tissue homeostasis, repair and disease, nor can it mimic the pharmacokinetics and -dynamics of drugs that can potentially treat cancer.

The properties of cells change in culture and there is no adequate in vitro model that supports the complete formation of hair follicles.

Reduction

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

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

My current licence uses an estimated total of 89,500 mice. However, 5 of the protocols in the current licence, which accounted for 4,000 mice, have been removed from the new licence and in practice the total number of mice used in the current licence is lower than estimated. The estimated total number of mice in the new licence is based on the assumption that the number of animal experiments performed by my lab (except for those involving the deleted protocols) will remain the same as in the previous 5 years, since we have used fewer mice than expected.



What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

In order to reduce the number of mice, we keep stocks of frozen sperm and embryos so that if a line of mice is not required for several months we avoid unnecessary breeding.

For chemical carcinogenesis experiments we use the same parental mice for the first and the repeat experiments, starting the repeats before the first experiment has been completed. This reduces the amount of mouse breeding required per experiment.

We encourage labs that have an interest in other tissues to obtain necropsy samples from our mice. This allows other researchers to obtain preliminary data without having to generate their own mice.

Prior to performing an experiment we perform statistical analysis to ensure that we use the minimum number of mice per group that will be informative. We aim to achieve a minimum power of 0.8, assuming statistical significance of $P < 0.05$.

When initiating skin carcinogenesis experiments I obtained advice on statistical analysis from REDACTED, who has written extensively on the design of animal experiments. I have previously attended a lecture entitled 'How many mice do I need for my experiment?' given by a staff member from the Wellcome Trust Sanger Institute. Members of my lab have attended the NC3R's Experimental Design Assistant workshop and their expertise is available. I can also consult our in-house statisticians at REDACTED for advice on experimental design.

Principles of good experimental practice, including randomisation and blinding, are inculcated into the laboratory. When planning experiments involving a new application - such as dosing with an anti-cancer drug - we begin with a pilot experiment before setting up the full experiment.

Although we can minimise the number of mice that undergo experimental procedures, in some cases a large number of animals have to be bred to obtain sufficient animals of the correct genotype. For example, we may need to activate a fluorescent reporter gene (mouse 1) by crossing it with a mouse expressing Cre recombinase in a particular cell type (mouse 2) and a mouse in which we genetically modify cancer susceptibility (mouse 3). In addition, some experiments require age and sex-matched mice, for example because skin tumour formation is influenced by the hair growth cycle and skin androgens. Our strategy in these cases is to use littermates that lack one or more of the transgenes as controls in the experiments.

What other measures apart from good experimental design will you use to minimise numbers?

Efficient breeding, as illustrated above, is an important way to ensure that the number of mice we use is minimised. We also perform pilot studies prior to embarking on a new set of experiments - this was the case when we initiated our current oral cancer studies. Computer modelling can enable us to predict the optimal time points for analysing experimental mice, thereby reducing the number of time points, and thus mice, to be analysed. We encourage sharing of tissue so that spare tissue from mice can be analysed to obviate the need for killing animals for tissue collection. We also have regular meetings of lab mouse users to ensure that lines of animals are not being maintained 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice are the lowest form of mammal that can be used to study normal and diseased skin and other tissues containing multi-layered epithelia. They are the only mammal in which transgene and knockout technology works reliably.

When generating transgenic mice we use inducible transgenes whenever possible. This reduces the time window when mice display a phenotype.

The different types of grafting experiment - for example, cells injected into the cheek and cells injected into skin chambers - are well tolerated and the end-points for the cheek injections are chosen to occur before the ability of the mice to eat is impaired. Tumours induced in the skin and oral cavity with chemical carcinogens are well established and again endpoints are chosen to prevent undue suffering.

When a new surgical or sampling procedure is involved, training on dead animals is first carried out.

When a new drug is being tested, an initial experiment is carried out with a small number of mice using the conditions predicted from the literature to be most effective, to ensure that the drug does not result in generalised adverse effects. The data from the first experiment then informs the design of subsequent experiments, which may involve larger numbers of mice.

In the case of administration of agents, the table of maximum dosing volumes and frequencies that is included in each protocol will be used. These are maximum volumes and numbers of injections by different routes that are normally well tolerated, assuming the substance being injected is non-toxic. These are the overall maxima for a single protocol.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The goal of our research is to understand homeostasis, repair and disease in adult tissues, and therefore confining our analysis to embryos is not appropriate. When performing in vivo imaging mice are subject to terminal anaesthesia but for all other experiments the duration extends to weeks and so terminal anaesthesia is not suitable. Mice are the least sentient mammals that are suitable for the proposed experiments.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?



All of the procedures in the licence are in use in my current licence and the most likely adverse events are not due to the procedures themselves, but to the genetic make up of the mice and the development of inflammatory disease and cancer. Strong communication is important between researchers and REDACTED staff to avoid animals going beyond their end points and to ensure that everyone involved is aware of what stage the animals are at in the study and what additional care is needed. In particular, when the chemical carcinogen used in the oral cancer studies is withdrawn mice can start to deteriorate as invasive tumours develop. The weights and overall condition of the mice is monitored at least twice a week and once any deterioration is seen the mice are monitored and weighed daily. A notice regarding monitoring is prominently displayed in the rooms where mice are kept to remind everyone what is required.

Throughout the period of the licence we will seek, and implement, ways to refine the procedures. For example, we have recently developed a nose cone for mice undergoing inhalation anaesthesia that facilitates examination and photography of the oral cavity and thereby reduces manipulation of the tongue and jaws. In the present licence we will ensure that animals in a protocol that involves regular handling, such as measuring tumour growth, will be handled prior to the start of the experiment, to ensure that the process does not induce unnecessary anxiety or stress to the animal. Mice being treated with a new compound will be monitored very closely in the early stages to ensure that any unexpected issues are caught rapidly. When performing oral gavage we will use a flexible plastic tube, rather than a metal one, to reduce the likelihood of tissue damage.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow regular updates on the Home Office website guidance on animal testing and research. We will read the specialist literature, such as the UFAW (Universities Federation for Animal Welfare) journal. We will attend events organised by the local AWERB. We will also check the NC3Rs website on a regular basis (nc3rs.org.uk).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I receive regular updates from the staff of the REDACTED in a variety of formats. Home Office recommendations are forwarded and we receive invitations to update meetings on campus. The Home Office Inspector is always very helpful in responding to queries face-to-face or via e-mail. Members of my lab also attend training at the REDACTED and we share best practice through meetings with other scientists who have shared research interests. REDACTED and lab members are kept abreast of the latest updates from NC3Rs.

Explain the choice of species and the related life stages

Mice have been chosen as the lowest form of sentient mammal that is of proven relevance to the pathophysiology of human tissues comprising multi-layered epithelia. The objectives of the project are to study homeostasis and disease in adult tissues and therefore the only experiments involving embryonic animals are either to manipulate gene expression in utero in order to study the effects postnatally (as in lineage tracing of different fibroblast populations) or to trace the origins of an early post-natal phenotype (such as postnatal skin fibroblast quiescence).





55. Host-symbiont ecology in REDACTEDs

Project duration

5 years 0 months

Project purpose

None selected

Key words

wild, rodent, microbiome, ecology, symbiont

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

This project aims to improve our understanding of why it is that animals in nature vary so widely in the symbiotic organisms that colonise them – their microbiome. Studies in laboratory animals as well as humans have shown that which microbes are present in the gut, and in what numbers, can have profound impacts on many aspects of animal biology – shaping immune responses, metabolism, and susceptibility to infection. And yet, we still know rather little about what determines the composition of these communities, how they pass from one animal to another, and their ultimate effects on health and fitness, in nature. In this project, we have three main objectives. First, we will use REDACTEDs to understand how a range of factors - including variable diet, aging and parasite infection - affect the REDACTED. Second, we also aim to understand how commensal gut bacteria, as well as viruses, pass from one rodent to another in the wild. This information is valuable as it may shed light on the pathways by which infectious organisms transmit among other mammals, including humans. It is also relevant for understanding the spread of pathogens infectious to humans that circulate in REDACTEDs (zoonotic organisms). Finally, we aim to explore whether the microbiome is important for animal health outside the laboratory (where most microbiome studies are performed) among REDACTEDs.

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

What are the potential benefits that will derive from this project?

This study will provide fundamental insight into why the microbiome of mammals is so hugely variable, and it's importance for animal health. It will show how factors relevant to



all free-living mammals, such as host genetics, diet and infection with parasites such as worms, impact these key microbial communities. As such, insight gained may ultimately be relevant for improving the health of humans, livestock, domestic animals and endangered animal species. It will also provide new information about why individuals within a population or community share particular bacteria and viruses, and the role that social interactions and space use play in this. This has value for our understanding of the epidemiology of rodent-borne zoonoses. Finally, part of our research focuses on how we can improve the experience of wild small mammals in scientific research, by designing new traps that prevent unnecessary captures and limiting capture time to the minimum required for those that do need to be caught. As such, in the long-run this work will benefit our animal study subjects.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

To do this, we expect to study approximately 1100 wood mice, 200 yellow-necked mice, 500 bank voles and 2000 wild house mice over a five year period.

Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

It is important for both our scientific goals and ethically that animals involved in this research stay in good condition, and any potential adverse effects are minimised. For most mice and voles, we expect to cause them only transient stress and mild discomfort associated with being trapped and handled, before they are released back to the wild at their capture site. Some animals will receive a drug treatment to reduce their natural parasite infection levels. If anything, we expect this to improve their health. While most animals will be set free as part of a capture-mark-recapture approach to studying wild animals, a small subset will be humanely euthanised allowing us to validate several of non-invasive measures we take on wild animals. For all animals involved in this study, the procedures we will use should only lead to mild and transient pain, stress or discomfort. Adverse effects should be very rare and we take careful steps to minimize the risk of these arising, through good trapping practice, rapid release of captured pregnant/lactating females, and careful monitoring of all animals in our care.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Since our goal is to understand how elements of an mammal's natural environment affect their microbiome over time, there is no alternative but to capture and sample live animals in their natural habitat.



Reduction

Explain how you will assure the use of minimum numbers of animals.

First, we will carry out careful experimental design including power calculations to estimate what number of animals are required answer the particular scientific questions. We use these estimates and our experience with REDACTED fieldwork to design field experiments that are not excessive, and use only sufficient animals likely to provide the required information.

Second, we will use appropriate and sophisticated statistical methods to analyse the data, that derive as much possible information from our study designs. This will mean we need fewer animals to answer a question than with more basic analyses.

Finally, we are pioneering the use of new technology to study REDACTEDs. This will reduce the number of unnecessary animal captures (by not allowing animals that we do not require for scientific reasons to enter traps), and replace capturing and handling animals with passive monitoring systems wherever possible.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

We will use good trapping practice to ensure animals involved in this work experience minimal welfare costs. This includes only setting traps when weather conditions are suitable, for the minimum time required (so animals cannot be trapped for too long), providing sufficient bedding and food to keep them comfortable and releasing animals in exactly the same location they were captured. We will also limit the frequency of trapping to minimize the potential adverse effects of animals being in traps too often. When animals have been caught and are in our care, we will carefully monitor them for signs of suffering, and react accordingly should this occur. We will choose methods of performing procedures that are as refined as possible. Finally, we have designed and will start using a new "Smart Trap" in this work, that prevents bycatch of non-target species and unnecessary recapture of individuals, and allows us to limit the time animals are held in traps to the minimum required for scientific objectives.



56. How does inflammation shape the course of disease in arthritis?

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

Rheumatoid arthritis, Inflammation, Immune cells, Therapy, Precision medicine

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Lay summary overview – Inflammation is the body's response to infection or injury and contributes to the healing process. In rheumatoid arthritis, appropriate control of inflammation is lost promoting joint disease. Importantly, the pattern of joint inflammation often differs considerably in rheumatoid patients to affect the rate of disease onset, the severity and response to therapy. Studies outlined here will define the mechanisms that account for these clinical differences. 'Inflammatory signatures' that reflect these various forms of joint disease will provide an opportunity to improve patient diagnosis and treatment.

Background to the problem – Cytokines are proteins that regulate inflammation and are considered major drug targets for the treatment of rheumatoid arthritis. These include the anti-TNF blockers. Here, adverse cytokine activities drive joint inflammation and disease progression. However, not all patients respond to these drugs and it is widely accepted that multiple, often independent, mechanisms contribute to joint disease. We propose that cytokine activities influence the type of inflammatory joint disease seen in rheumatoid patients. Thus, experiments aim to understand the inflammatory mechanisms that determine the course of arthritic disease.

Questions we aim to address – We have characterised a cytokine-controlled mechanism that directs the pattern and severity of inflammatory arthritis. To build on these findings we will answer the following questions:



- What are the inflammatory mechanisms that drive chronic joint disease?
- Why do patients with rheumatoid arthritis display different forms of joint inflammation?
- How do the activities of particular cytokines affect the type of joint inflammation observed?
- Can inflammatory signatures that reflect these differences in rheumatoid arthritis improve patient diagnosis, clinical management and response to therapy?

The Experimental approach – Experiments will establish how inflammation promotes the various forms of joint inflammation observed in patients. Here, differences in inflammation refer to the composition and specific organization of infiltrating immune and structural cells within the diseased tissue. We will examine the response of these cells to cytokines. Using cell-based systems, mouse models of arthritis and clinical samples including ultrasound-guided tissue biopsies from rheumatoid arthritis patients, studies will identify response patterns that predict the course of disease. Specific approaches will explore differences in gene expression, the mechanisms that affect these gene changes and the impact of these events on joint 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.

What are the potential benefits that will derive from this project?

Our objective is to aid patient diagnosis, treatment and cure by identifying the mechanisms that dictate the course of joint disease and inflammation. Studies will help identify how patients with the same condition display fundamental differences in the presentation of local joint disease. Our aim is to deliver long-term patient benefit by improving the clinical management of disease and potentially enhancing the likelihood of clinical cure. The identification of novel diagnostic tools that predict differences in disease activity or the presentation of disease will support clinical decisions relating to the selection of the most appropriate therapy for an individual patient group.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Mice – both wild type mice and genetically manipulated strains that alter the capacity of mice to mount to an immune response.

Experimental Numbers – 4,250 (over 5 years).

Breeding Numbers – It is anticipated that a maximum of 3000 animals will be bred under this licence.

Predicted harms

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



In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Expected level of severity – Mild to Moderate

Outcome – All animals will be killed

Adverse effects – All animals will be immunised with agents that will either promote arthritis (causing bone and joint damage and inflammation) or alleviate the signs and symptoms of disease. Following the onset of disease this may result in a loss of mobility and some mice may lose their normal exploratory behaviour. These changes are however short lasting and typically go back to normal once the initial joint swelling has reduced. In some instances mice may encounter an adverse response to reagents administered to promote arthritis. This may include mild skin irritations at the site of injections, or more pronounced changes in wellbeing or health (e.g., fever-type responses or changes in normal animal behaviours).

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

The immune processes responsible for the development of arthritis likely occur before the presentation of clinical signs and symptoms of joint disease (i.e. when the disease may not be overtly apparent to the patient). These early warning signs will cascade the escalation of inflammatory processes leading to the onset of arthritis – affecting the severity, the rate of disease progression and the response to therapy. Unfortunately, this initial phase of arthritis is difficult to track in humans and remains subjective. At present there is no alternative to using the *in vivo* models described to achieve the experimental objectives outlined in this licence application.

Reduction

Explain how you will assure the use of minimum numbers of animals.

We routinely discuss our research with clinical colleagues to ensure we ask meaningful questions. Our previous research has yielded a large amount of data, and archived samples for training purposes and informed decisions on the best study design (e.g., selecting specific time points within the model). However, our laboratory is now using exciting new methodologies that enable us to generate more information from fewer mice. These include the introduction of next generation sequencing techniques to capture whole genome changes in gene regulation and expression. Moreover, we also use comparable datasets derived from human clinical studies to verify our mouse findings, and this approach often informs new questions that can be tested in our experimental model systems.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.



Inflammation is a complex process that is determined by a close communication between immune cells and cell resident to the site of disease (e.g., the inflamed joint). Many of these responses are also controlled outside the inflamed joint. This necessitates the need for a model system that can reflect this high degree of complexity. Mice provide an excellent model system in which to study the relationship between the immune system and arthritis development. Mice are well characterised immunologically, and their immune systems closely resemble those of humans. In addition, several genetic modified mice lacking various immune molecules/cells have already been generated and provide an ideal opportunity to perform detailed analyses of immunological function. In this regard, our research has now identified three mouse strains that closely resemble the various forms of joint disease seen in humans. We are therefore uniquely placed to realise the ambitions of this study, and have the capacity to draw upon information derived from both human patients and mouse models.

We (in collaboration with others) have refined these models through discussion with clinical colleagues, and explored new methodologies that enable us to optimise experimental questions. This has been achieved through an increased understanding of the model, with specific regard to how immune cells behave and the time points that immune cell are critical for specific aspects of disease, this ensures that only high value time points are chosen and mice are not subjected to needless procedures at non - relevant time points. The Introduction of new imaging techniques can lead to a number of refinements . For example, previous monitoring protocols have included excessive handling of the mice (e.g., measurement of joint swelling using callipers). This causes distress and anxiety to the mice housed together and will invariably moderate immune effects linked with stress. The use of these alternate imaging techniques means that certain measurements can be conducted only once at the end of the study once the animals have been killed.

In addition the following steps will be utilised as best practice:

We will Ensure that good practice is observed with training, supervision, assessment of competence, Continuing Professional Development and skill refresh on a yearly basis, seeking advice both internally and externally as necessary.

We (in collaboration with others) have refined these models through discussion with clinical colleagues, and explored new methodologies that enable us to optimise experimental questions. This has been achieved through an increased understanding of the model, with specific regard to how immune cells behave and the time points that immune cell are critical for specific aspects of disease, this ensures that only high value time points are chosen and mice are not subjected to needless procedures at non - relevant time points.





57. How neuronal activity links sensations, perceptions and actions

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neuroscience, Sensory coding, Decision making, Somatosensory cortex, Neural circuits

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 is the aim of this project?

The world around us is full of temporal structure: things happen in specific sequences and patterns over time, and this information is essential to our ability to make sense of our environment and, ultimately, to survive. The aim of this project is to use wild type and genetically altered mice to record and manipulate neuronal activity while animals carry out a task guided by sensory input, in order to understand how neuronal activity underpins the animal's capacity to sense and identify temporally patterned sensory signals and decide on an action in response.

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

Why is it important to undertake this work?

The project will illuminate how neuronal circuits give rise to sensory-guided behaviour. Progress in this area is central to our understanding of how mental function arises from the healthy brain, and how it is affected by disease. Such an understanding is fundamentally needed to drive improvements in the treatment of neurological disease.



Our understanding of how neuronal activity underpins sensory perception and drives decisions and actions remains limited, particularly in the cerebral cortex, a region that is key to the formation of sensory percepts, decision making and cognition. Sensory processing and its translation into decisions is affected in many pathologies of the cerebral cortex, e.g. developmental disorders such as autism or schizophrenia.

By allowing measurement and interference with neuronal activity while mice perform a task on which they have been trained, we will be able to directly link neuronal activity with task performance, and this will provide a highly powerful experimental framework.

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

The primary objective of this work is to generate new scientific knowledge. Our research will advance understanding of how neuronal circuits give rise to behaviour, and specifically how brain activity underpins the capacity to reach decisions based on sensory signals. Accordingly, the main scientific output will be in the form of conferences and of publications on this topic in open access peer-reviewed journals.

As part of this work, we will develop experimental protocols allowing the measurement of neuronal activity during the performance of sensory processing and decision making. Insights into procedures for high-data-yield mouse behavioural training will be shared with the academic and industrial research community through an existing NC3Rs working group and the publicly available peer-reviewed publications that will result from it.

Our understanding of information processing at single-neuron resolution in the healthy and diseased brain remains relatively limited, particularly in the cerebral cortex, the region underlying percept formation, decision making and cognition. The outcomes of this project will have implications for basic research beyond improving our fundamental understanding of information processing in cortical neurons. Many devastating and complex diseases of cortical circuitry, such as autism spectrum disorders or schizophrenia, affect poorly understood aspects of circuit organisation and disrupt higher sensory function, including the processing of sensory sequences and the resulting ability to make decisions based on sensory evidence. Reaching a better understanding of how cortical circuits achieve these functions is important for illuminating how the healthy brain constructs our experience of the world and how the functional consequences of brain disorders can be ameliorated.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short term, conference communications and publications will be addressed to academics and other researchers (e.g. in industry), and this will be the earliest form of benefit. Other potential benefits primarily affecting academic and clinical researchers include:

adoption of procedures refined during the course of this work (short-term);

adoption of our behavioural tasks as an assay for testing sensory-decision function in the cortex of healthy mice and disease models (medium-term);

improved understanding of brain function in disease models (medium to longer-term; any potential extensions to human patients would occur long-term).



We will also target public communication as we have done in the past: our group has a very strong track record of public engagement (school visits, lab demonstrations, public conferences, media appearances). This will occur over the short- to medium-term.

How will you maximise the outputs of your work?

Dissemination of new knowledge through conventional scientific primary publications

Deposition of data and of software code in public repositories and on journal websites
REDACTED

Collaborative publication of best practices in mouse training, arising from high-yield rodent behavioural experiments working group

Species and numbers of animals expected to be used

- Mice: 2900

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mice used only on Protocol 1 will only undergo breeding and maintenance. Those will be the majority of animals on this project. Experimental animals used on Protocol 2 will usually undergo behavioural training on a sensory-guided discrimination task, most often including (i) an implant surgery (sometimes including cranial surgery for implantation of devices such as electrodes, cannulas or a cranial window), (ii) daily head fixation using an implanted device, and (iii) a form of water or food control or restriction.

Implant surgeries will last about 1 hr. Daily training sessions will typically last between 30 min and 1 hr, but no more than 2 hr including all associated preparation. Animals will be given opportunities to top up their water intake after the end of the session and will not be left for more than 24 hr without access to water.

After surgery, animals will not be kept alive for more than 8 months. Training on a task will not last longer than 6 months. Training may be combined with recordings involving either electrophysiology or imaging, but not both.

Some animals will undergo anaesthetised recordings at the end of their use. Although experiments under anaesthesia do not allow one to test relationships between neuronal activity and perception, they can help establish fundamental properties of neuronal responses to sensory stimulation. Stimulation is highly controlled thus allowing for greater analytical power and reproducibility.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.



Pain and discomfort from tissue being taken for biopsy (duration: transient, seconds to minutes). Some genetically modified animals may have the potential to develop a harmful phenotype, e.g. tumours, neurological signs, after a certain age (cannot be fully predicted but all types of mice will be monitored for side effects). Some genetically modified animals may have an altered immune system. Blood loss and hypothermia could occur during surgeries (duration: tens of minutes; monitored in real time during surgery). Pain, physiological adverse effects (inflammation, infections, dehydration, weight loss) or distress could arise during surgery recovery and training, manifested in physiological or behavioural alterations (duration: up to a few days; monitored continuously).

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

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

Mild: approximately 86%.

Moderate: approximately 14%.

What will happen to the animals at the end of the study?

- Used in other projects
- Killed

Replacement

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

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

There is no realistic alternative to in vivo data collection for understanding how the brain generates sensory function. We use electrophysiology and optical (two-photon) imaging to record activity in vivo because other techniques cannot provide the resolution needed to analyse the activity of individual neurons. We combine recordings with behaviour because we need to understand how neuronal activity is modulated by brain and behavioural state, and how activity contributes causally to behaviour.

What was your strategy for searching for non-animal alternatives?

Computer simulations: these can help guide predictions based on known data.

In vitro experiments: these can provide basic information about how neurons and synapses work, about neuronal circuit anatomy and connectivity, and about how neuronal activity alters the signals (e.g. fluorescence from genetically encoded calcium indicators) that we use to monitor it.

Reuse of experimental data in repositories: as part of the movement towards open access dissemination of results, a growing amount of neuronal data collected in vivo from animals carrying out sensory-guided tasks is being made publicly available.

Why were they not suitable?



Computer simulations are not sufficiently constrained by experiment and cannot take into account unknown factors crucial for brain function, including changes in brain state or context for a given behaviour: to simulate the brain, we will need much more validated experimental data to generate meaningful output. Thus we cannot yet replace observation with simulations.

In vitro experiments cannot reproduce the behaviour of an intact neuronal circuit. We do use in vitro work to determine neuronal circuit connectivity and to check the expression levels and functioning of the sensors we use to monitor activity (e.g. calcium indicators). This is important to improve interpretation of our in vivo experiments and reduce the need for further such experiments.

Data currently available in repositories do not yet reproduce or approximate the experimental conditions described in this project. However, during the project's lifetime we will monitor openly available data and will reconsider our experimental plans whenever there is the option to reanalyse suitable data rather than generate it anew.

Reduction

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

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

We have used statistical methods (power analysis) to estimate how many neurons we need to record in order to establish the proportions of neurons that play different roles in the brain when an animal is performing a sensory discrimination task. This gives sample sizes on the order of 400-500 neurons for each tested brain area. From this estimate we have derived experimental mouse numbers by taking into account the number of neurons that we can typically observe in a session, the proportion of neurons that are found experimentally to be responsive in an experiment, and the actual success rates for mice learning the task and allowing us to generate data. The resulting numbers of experimental animals are also consistent with those actually reached empirically in our previous licence.

We have also estimated breeding numbers for maintaining colonies of mice genetically altered to express indicators of neuronal activity, or molecules that allow us to manipulate activity. The majority of offspring with an appropriate expression genotype will be used for the experimental protocols.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Three key aspects of the experimental design are (i) the longitudinal use of animals to record activity at successive sessions during training, leading to a drastic reduction in the number of animals used; (ii) the development of training protocols that can generate several hundreds of trials (repetitions) of performance per session, enhancing statistical power; and (iii) the use of state-of-the-art techniques for neuronal recording and manipulation that allow data collection from many neurons in parallel during task performance. These, coupled to actual calibration of empirical success rates as described



in the previous section, have both been taken into account in our estimate of numbers. All estimates involve the use of online tools for power analysis.

What other measures apart from good experimental design will you use to minimise numbers?

Efficient use of animals bred both under this licence and under the authority of other licences within the establishment that allow for the breeding and maintenance of mice expressing genetically encoded reporters and effectors of neuronal activity. For example, we will avoid ordering additional wild type experimental animals whenever surplus mice can be obtained locally.

Longitudinal data collection will not only include functional experiments as described above, but will also include tissue collection for histological analysis and any novel reagent testing.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The project requires a valid model for studying neuronal activity in the mammalian brain and how it gives rise to behaviour. Traditionally, this type of research was carried out in primates, the species most closely related to human. However, rats and mice can perform behaviours previously thought to be only within reach of primates. The networks of neurons underlying sensory processing have a fundamental organisation that is remarkably conserved across different mammals, so that rodents can provide a good experimental model for their study. At the same time, rodents have perceived lower sentience than primates and provide a standardised model for laboratory biology. Much knowledge has been built up over the years on basic mouse brain anatomy and neuronal properties, and new work can build on this, avoiding the need to re-establish fundamentals anew in a different species. Tools for genetically based targeting of neurons in mice permit more powerful and precise experiments.

Consideration for welfare of the animals is integral to the project's design. Animals will be given pain killers peri-operatively and closely monitored after surgery for signs of pain or distress. Animals routinely learn to perform the behavioural task without evidence of distress or ill health. We will ensure animals can reach the desired performance by combining incentives (positive food or liquid rewards) with the least severe form of restriction. A training approach in stages will be used to acclimatise animals and minimise stress. Animals will be group-housed wherever possible, with exceptions to this rule being when required for purposes of welfare (e.g. post-operatively) or when a single animal needs to be monitored for water and food intake.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?



The project's aims interrogate the relationship between neuronal activity and behaviour in the intact, embodied brain of a mammal aware of its sensory environment. This requires animals to undergo training in a sensory-guided task after having undergone an initial implant surgery. The need for a longitudinal period of training implies that implants must be stably mounted on a stably sized skull, hence the need to wait until the animal has reached adult size before starting training procedures.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Multiple refinements to procedures during and after surgery and to mouse training will be incorporated.

Animals will be given pain killers and closely monitored after surgery for any signs of pain or distress.

The temporal separation between surgery, training and other procedures has been designed to ensure that any adverse effects can be monitored, that animals recover from any step before undergoing the next, and that accumulation of effects is limited. Training incorporates extensive monitoring and habituation. Animals are given rewards from the outset of training, at the step where they first become familiarised with the experimenter; rewards are combined with the least severe form of restrictions to encourage the animal to learn the task. Animals will have enrichment available in their home cage. These refinements are based on best practices shared by other labs as well as our own experience performing similar experiments under a previous licence.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will continuously refine our training procedures to follow best practice in this area, which is a very active one at the moment; e.g.,

Guo et al (2014) Procedures for behavioral experiments in head-fixed mice. PLoS One 9: e88678.

Burgess et al (2017) High-Yield Methods for Accurate Two-Alternative Visual Psychophysics in Head- Fixed Mice. Cell Reports 20: 2513-2524.

Goltstein et al (2018) Food and water restriction lead to differential learning behaviors in a head-fixed two-choice visual discrimination task for mice. PLoS One 13: e0204066.

Our participation in the NC3Rs working group mentioned elsewhere, through which we will disseminate any refinements throughout the community, also puts us in an excellent position to hear about and adopt any relevant updates to refinements developed by other researchers.

Surgical procedures will be carried out according to the published LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017).

How will you ensure you continue to use the most refined methods during the lifetime of this project?



Several avenues:

Participation in NC3Rs working group on 'high-yield rodent behaviour', through which we share best practice in this area;

NC3Rs bulletins and newsletters;

News and information provided by local NACWO/NTCO;

Scientific meetings with presentations on mouse behaviour and training.

Explain the choice of species and the related life stages

The project requires a valid model for studying neuronal activity in the mammalian brain and how this activity gives rise to behaviour, in an animal interacting with its environment and where the causal contributions of activity to behaviour can be experimentally dissected. Mice can readily learn to perform sophisticated sensory-guided behaviours, and the basic neuronal circuits for sensory processing and decision making are remarkably conserved across mammals. Mouse-based experiments can build on a wealth of knowledge concerning the basics of neuronal circuitry and anatomy, implying that reaching a certain level of insight requires less additional experimentation. Tools for genetically based targeting of neurons are highly developed in mice and permit powerful and highly reproducible experiments measuring and modifying neuronal activity.

Animals typically need to undergo weeks of training after undergoing an initial implant surgery. Thus, before beginning procedures, the mouse must have attained a stable size and behavioural repertoire. The project addresses the functional study of neuronal activity but does not require tracking changes in activity during brain development. For these reasons, the work involves animals that have reached adult size.





58. Identification of mechanisms that control resource allocation in mammals

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

To understand how the baby communicates with the mother during life in the womb and during lactation (milk suckling), to obtain the food and other resources it needs. We want to know how genes (the set of 'instructions' in our cells that that makes us what we are) and what surrounds us (the environment) affect this communication between mother and baby, and how these processes affect our health when we get older.

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

Why is it important to undertake this work?

We know very little about how and when a baby sends signals to the mother to get the food it needs to grow in the womb. The placenta is a specialized organ of the baby, whose function is to exchange food and gases between mother and baby. It is attached, on one side, to the womb through blood vessels and maternal cells, and on the other side, to the umbilical cord - that takes food and gases to all organs of the baby, including the brain. At birth the baby is delivered, followed by the placenta. Remarkably, the baby is somehow able to ask for more or less food by communicating its needs to the mother, through the



placenta. Similar communication systems (of the baby to its mother) also exist through the period of breast feeding. We call these systems "resource allocation" systems. We know that genes play a role in this communication, as well as the environment too, but precisely how this occurs is not well known.

Studying resource allocation processes is important for two main reasons:

Suboptimal resource allocation is linked to developmental problems, from poor growth in the womb, called fetal growth restriction (FGR), to other pregnancy complications or 'diseases', such as pre- eclampsia - increased blood pressure in the mother- and gestational diabetes - elevated levels of sugar in mother's blood that go away after the baby is born. For example, FGR is a serious complication of pregnancy, which can result in the death of the baby in the womb (stillbirth) or soon after birth, and significant disease in early childhood and adulthood. FGR is thought to affect ~10% of all human pregnancies and contribute to about 1 in 4 stillbirths. Furthermore, newborn ill health amongst survivors of FGR pregnancies is significantly increased: 25% to 40% will require prolonged assistance with their breathing, and 4-6% will develop a severe gut disease called necrotizing enterocolitis. Long- term childhood disease for survivors of pregnancies affected by FGR is also increased. A total of 8% of all growth-restricted survivors will have some degree of brain impairment.

Low birth weight is also associated with increased risk of disease in later life (so called development programming of adult disease).

There is no effective treatment for FGR, despite the current clinical, social and economic burden (caring for survivors of an FGR birth in newborn intensive care costs the UK National Health Service at least £420M per year). Early diagnosis can allow intensive monitoring of the affected woman and baby but this only informs the crucial decision about when to deliver the baby (preterm delivery is itself a cause of poor pregnancy, newborn and childhood outcomes). The current REDACTED guidelines on care of pregnant women identified research on FGR as one of five research priorities. There is a considerable lack of drugs available, or even in the pipeline, to deal with serious pregnancy problems such as FGR: in the last 20 years only one new class of drugs has been licensed for pregnancy applications and no new drug developed primarily for pregnancy applications is currently in clinical trials.

In the past few decades, it has become evident that healthy living and aging is determined not only by lifestyle choices but by the interaction of the genetic programme with the environment. Such interactions can occur at any stage of development, from embryonic life to adulthood and affect future generations through effects on developing germ cells (cells that give rise to sperm and eggs). Our understanding of gene-environmental interactions, in particular those related to parental resource allocation to offspring, are poorly understood.

Control of resource allocation is therefore a major component of growth and development in species like us (placental mammals) and is a key underlying cause of programming of adult disease. This work is important as it will address an enormous gap in knowledge in this area of study and will provide unique animal models for the study of effects, over several generations, of environmental cues on development and disease, whilst providing new drugs to treat pregnancy complications and long-term health outcomes (such as diabetes and obesity).

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



For every 100 human newborns, 10 to 15 do not grow well in the womb. Those babies are called fetal growth restricted (FGR) babies. The National Health System (NHS) spends vast amounts of money on treating the complications of FGR pregnancies in newborns and infants.

Moreover, being small at birth increases your risk of becoming diabetic and having heart problems in old age. At least 10% of the NHS budget is spent on treating diabetes of older people (type 2 diabetes) alone. Given that the quality of life in later life, and how long we expect to live depends to a certain extent on the events that occurred during pregnancy, there is a strong argument for the use of animals in the advancement of knowledge in this area of research. The overriding benefits to using mice to answer these questions are their short generation time and the ease of manipulating environmental conditions, but above all the possibility of altering their genetic make up. Resource allocation is under tight control of the genes in our cells, so the study of genetically modified models is likely to provide important new insights into this fascinating field of developmental programming of adult disease.

This project addresses two main questions: how do babies use maternal resources optimally, and why are babies that don't use those resources properly more likely to develop illnesses such as diabetes and cardiovascular disease? By answering these questions we should be able to come up with a solution to delay, reverse or prevent diseases in the womb, and their long-term effects (in the first instance in mice, which can then be tested for application to humans).

Specifically, the expected benefits of this project are:

Generation of three new mouse models of human pregnancy complications and/or metabolic dysfunction (that is, diabetes and obesity)

Identification of two new mechanisms underlying major pregnancy complications (fetal growth restriction; pre-eclampsia, which is a disease in mothers that is characterized by increased blood pressure and abnormal substances in urine; and miscarriage, which is when a baby dies in the first six months of pregnancy)

Elucidation of two new molecular mechanisms that can explain why and how baby growth impairment is linked with diabetes in later life

Assessment of two potential drugs in mice for the treatment of fetal growth restriction/pre-eclampsia and/or type-2 diabetes

Major outputs will be scientific publications (expected to be around 8) describing the findings of the research with the above mouse models.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

We are investigating issues related to the recent “explosion” in the number of people affected by obesity and diabetes, which cannot only be explained by our genetic makeup. We know that it is the interaction between genes and the environment that ultimately causes diabetes and obesity. However, we know very little about how the environment provokes changes in the behaviour/activity in our genes. We think that the answer to this



lies in natural “chemical” signals that are physically placed or removed from our genes (this is called epigenetics, literally meaning on “top of our genes”). We will induce changes in these chemical signals (epigenetic marks) in mice that are exposed to sub-optimal nutrition or other adverse environmental factors (e.g. drugs, hormone-disrupting chemicals, manipulation of their eggs [oocytes] in a test tube), measure metabolic and cardiovascular characteristics in these mice, and see how these epigenetic marks contribute to the phenotypes of their offspring and of subsequent generations of mice (grandoffspring and great-grandoffspring).

The major benefit of our research will be the provision of new knowledge and the better understanding of growth diseases (including pre-conceptual causes) and associated developmental, physiological and metabolic complications. Other potential benefits relates to the early diagnosis of growth and metabolic-related diseases and the design of new therapies to prevent and cure these diseases.

Specifically, in the short term this project will identify the extent to which resource allocation is under the control of our genes, and how the environment interacts with the DNA to modulate these processes. In the medium term, the knowledge gained will assist in the design of drug or dietary interventions for the improvement of common pregnancy complications and metabolic complications (diabetes and obesity). In the long term, we hope that drugs or nutritional interventions that have been tested in animal models will inform human intervention trials.

How will you maximise the outputs of your work?

The main output of this work is in the form of scientific publications (primary research papers, literature reviews, book chapters). We will seek to file patents relating to discoveries with potential clinical impact. All datasets will be made publicly available upon publication. Our tissue bank will be shared with colleagues and/or used for future/other work.

Our work is highly collaborative. This project license will allow us to consolidate previously established collaborations, as well as setting up new ones. We feel that collaborative work is the fastest way to achieve progress and to maximize resources that can be of great benefit to groups of people working closely together.

Dissemination of our work and knowledge, beyond our research field, will be carried out to the lay public in the form of press-releases and attendance at public engagement events, as we have done in the past, having seen our work widely covered in the national and international press, and having participated in various science festivals/public engagement sessions.

Species and numbers of animals expected to be used

- Mice: 9840

Predicted harms

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



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically in the 'parental' experiments we will perform dietary manipulations (i.e. diets with varied constituents) to the mother and/or father, genetically altered or not, at any stage of their reproductive age, and analyse the effects of nutrition in the offspring during intra-uterine life. We will also be able to apply a physiological stress, for example placing pregnant dams in an environment with low oxygen concentration, and follow the outcome in offspring, or a specific therapy to correct a specific pregnancy complication arising from a genetic manipulation. These parental experiments were devised to allow for the effects of environmental exposures, physiological stresses or therapies, that were applied to parents, to be followed in the offspring for several generations (so called transgenerational study). Within these experiments the main surgical procedure that might be done in some animals is the narrowing down of the maternal vessel that provides blood to the fetuses, with the aim of reducing the blood flow by 40%. After recovery from surgery, other procedures are terminal in some animals, for example, we will follow the transfer of nutrients from the mother to the fetus by measuring the accumulation in the fetus and placenta of a radioisotope injected into the jugular vein of a terminally anesthetized pregnant dam. Parents may also be injected with substances, or drugs delivered by small devices called 'osmotic minipumps', which are implanted under the skin and can slowly release the drugs as they expand in volume as water is absorbed. We will study the effects of these substances or therapies in the development of the offspring but also in the parents themselves.

In the 'offspring' experiments, we will follow and study the offspring, after they are born and all the way until they become adults, obtained from parents that have either undergone experiments or not (the latter act as controls). Under these experiments we will study the effects of diet, physiological stressors and will apply potential therapies (via minipumps or injections) to correct health problems, primarily related to growth and metabolism (e.g. how mice handle high sugar in the blood stream).

The vast majority of mice to be used in this project will experience procedures that will only cause mild and transient discomfort. Importantly we also expect that health problems caused by manipulation of genes will not cause any lasting harm and will be sub-threshold or mild. Examples of procedures that will only cause mild level of non-lasting suffering are blood sampling, live imaging (that require some level of animal restraint), housing in specially designed cages, called metabolic cages, that we use to measure water, food intake, and collect urine and faeces, and tests devised to understand how food is being converted into energy, where either sugar or olive oil will be administered through a tube leading down the throat to the stomach, or insulin by injection in the area that contains the abdominal organs (or peritoneal cavity).

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The major surgical procedures refer to insertion of fertilised embryos into the uterus (or womb), vasectomy (surgery that blocks the sperm to reach the male reproductive fluid) in male mice, and the narrowing down of the maternal vessel that provides the blood flow to the fetus, all of which may cause transient pain (however medication that relieves pain will be provided). There is a very small chance of infection as all the surgical procedures are done under the most stringent surgery guidelines.



The major experimental procedures that are not done as a terminal step (i.e. the mice will die after being given lethal dose of anaesthesia) relate to: a) the implantation of small mini-pumps to deliver drugs or other substances to the blood stream at a constant rate; b) the administration by oral gavage (through a tube leading down the throat to the stomach) of specific amounts of nutrients (sugar, olive oil), with small amounts of blood being taken from mice at regular intervals to see how long it takes for those nutrients to clear from the blood; c) injection of hormones like insulin, drugs or other compounds. These procedures will not lead to lasting harm, but instead are associated with transient pain and discomfort, which are mitigated by the prompt use of medication that relieves pain. In rare occasions, infection or inflammation at the sites of injection or implantation may occur.

Certain procedures such as those related to measuring blood pressure, imaging, or the placement of mice in a low oxygen (known as hypoxia) chamber, may require a period of adaptation. The adverse effects are mainly stress-related that can be alleviated by a short period of training.

The major abnormalities we are expecting to see from genetic manipulations will only affect a small number of mice, and are likely to arise during fetal life. These include fetal loss, prematurity, and developmental defects. The most common effects we expect to see in the genetically altered mice are related to growth (of the whole animal and/or specific organs) metabolic (not responding well to high sugar levels, not responding well to the action of insulin). Nutritional or genetic manipulation of a number of mice may render them pre-diabetic, diabetic, or obese. When studying how these animals respond to tests aimed to evaluate how they respond to insulin (the so called insulin tolerance tests), some mice may develop exceedingly low levels of sugar in their blood (called an hypoglycaemic event) which need to be counteracted with an injection of sugar.

Ill health (normally associated with piloerection - which is erection or bristling of hairs due to the contraction of small muscles at the base of hair follicles that occurs as a response to cold, shock, or fright- subdued behaviour, loss of appetite and weight, inactivity, shortness of breath, diarrhoea) will be a very rare event in mice used under this licence. We apply very strict criteria to define the health status of the mice, and depending on their wellbeing only mice will either be treated or 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 species)?

We expect over 90% of mice to experience only mild and transient discomfort, with less than 10% experiencing moderate suffering.

What will happen to the animals at the end of the study?

- Kept alive
- Used in other projects

Replacement

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



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

We want to understand how a fetus communicates growth and developmental needs to its mother through the placenta. Mice are the most suitable model for this project because of similar placentation processes to humans and because it is the most amenable species to genetic manipulations *in vivo*. The physiology of the mouse placenta, mouse growth and metabolism shares important features with humans, and can thus be used to model human disease. Mice also offer unique advantages when performing nutritional studies during pregnancy and metabolic studies during their life-course.

What was your strategy for searching for non-animal alternatives?

Observational studies in the animal are an absolute requirement for the successful completion of our aims. Where possible we will follow up these observational studies in the animal with non-animal cellular systems to gain mechanistic insight into specific cell signalling pathways.

Why were they not suitable?

The scientific understanding of placental, maternal and fetal signals that control resource allocation requires the study of whole organisms. For our work we need to apply physiological assays in a living mouse (for example placental nutrient transfer assays; or uterine blood flow measurements) in order to understand the interplay between the mother, the placenta and the fetus in growth control. While *in vitro* systems (that is cells cultured in a dish) are potent tools for dissecting molecular pathways, they cannot address the kind of questions we are asking at the level of whole body complexity. We need to study how different cell populations behave and interact as part of a complex environment in a living animal. Each tissue type, for example fat cell, placental cells, liver, brain, heart cells sends out and respond differently to the signals present in the peripheral blood system such as it occurs in the whole living animal. This level of complexity can't be addressed in cell culture based experiments.

However, we will run experiments in cells as much as we can, in order to optimise better experimental design and to achieve a reduction in the number of animals required. For example, once we identify a target gene that we suspect might be involved in growth control, we will conduct gene knockdown experiments in cell lines (using technology, called siRNAs and/or CRISPR, that is based on the use of RNAs to guide molecular scissors to specific genes to cut them out) and assess the effects on cell proliferation parameters. We have and will continue to derive mouse embryonic fibroblasts (MEFs) (primary and/or immortalised) from many of the mutant mice we generate or hold. MEFs are particularly valuable for dissecting molecular pathways downstream of the gene of interest. We will also use appropriate human term placental samples from the REDACTED study, an ongoing study in our Department (REDACTED, which comprise serial ultrasonic data and maternal blood at 12, 20, 28 and 38 weeks of gestational age and placental samples, at birth, from 4000 unselected first time mothers) for translating our mouse studies into human populations. We will also use human adipose tissue samples from collaborators to provide translation of metabolic findings in mouse models of nutritional manipulations into human obesity and diabetes.

In addition, to translate fundamental biomedical research into clinically useful products or protocols, it is essential to test key hypotheses in pre-clinical models, such as the ones we will be developing during the course 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 number of animals to be used are usually set with the help of a mathematical model, which we call a 'power analysis' method. It allow us to calculate the minimal number of animals we need to use, for a particular experiment, in order to find differences between groups of animals, whilst making sure that those differences we may find are not due to chance.

When appropriate, we conduct pilot studies to ascertain the variability of the data before applying 'power analysis' statistics. In some instances, previous experience (ours, or from the literature) is used to select sample sizes.

For this project we will also verify our estimates against new experimental tools that came to our attention within the National Centre for the Replacement and Reduction of Animals in Research (NC3R) website (<https://nc3rs.org.uk/experimental-design-assistant-eda>).

In general terms (of the numbers of animals required), we expect that ~ 8-12 animals per treatment group is sufficient to obtain the desired results for most of the physiological assays (for example, glucose tolerance test, which is a test we use to know how well the body clears an unhealthy amount of sugar we may have in blood; or placental transfer assays, which allows us to follow how well nutrients go from the mother to the growing baby across the placenta). For growth kinetics we use statistical modelling based on historical growth data we have accumulated over the years, in collaboration with local statisticians, which allow us to obtain an unprecedented level of accuracy for detecting onset of growth restriction and patterns of growth rates, whilst using the lowest number of animals possible.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Reductions in animal usage will be achieved through the use of *ex-vivo* methods (that is, organs or cells taken out from mice) and/or *in vitro* methods (that is, cells growing in a dish) and careful experimental design that includes power analyses and optimized breeding strategies (to reduce the number of mice that don't carry the genetic alteration and therefore do not show what we are looking for).

We will keep the number of animals to a minimum by making as many observations and measurements as possible on individual mice and by removing as many tissues as appropriate for *ex-vivo* studies (i.e. we collect most tissues and organs at post-mortem even ones that are not required at the time for any particular study - from this tissue bank, we are able to facilitate later studies without the need for additional numbers of animals, thus significantly reducing the need to use more animals).



In the past we developed *ex-vivo* and *in-vitro* models, such as primary culture of placental endothelial and pancreatic cells, which are the cells found in blood vessels and pancreas, respectively, and used established cell lines by others to replace or complement the studies using live mice (i.e. *in vivo*) for particular aspects of the work, and will continue to do so in this project. Replacing the use of mice for the core fetal growth studies will not be possible as we interrogate, for example, the communication between the placenta and the fetus, and the transfer of nutrients from the mother to the fetus through the placenta. However, by conducting some experiments *ex-vivo* and *in-vitro*, which are aimed at finding underlying cellular/molecular mechanisms, we are able to replace, at least in part, the use of animals for that specific purpose.

What other measures apart from good experimental design will you use to minimise numbers?

We collaborate with others in the placenta, growth control and developmental programming fields, sharing tissues, mouse embryonic fibroblasts (MEFs) and experimental results from our various genetically engineered mouse knock-outs. We are part of local schemes (i.e. Strain Enquiry list) that share information about strains, surplus of mice and potential tissue sharing.

Wherever possible we adopt non-invasive procedures enabling repeated measurements in the same animals (e.g. Time Domain Nuclear Magnetic Resonance, or TD-NMR for short, which is a machine that allow us to know very precisely how fat or how lean a mouse is) thus minimizing number of animals required.

Moreover, the culture in a dish of small sacs of fluid found on the outside layer of the ovaries, called follicles, which contain immature eggs (oocytes), and oocytes surrounded by specialized granulosa cells, called cumulus cells, will allow us to expose oocytes to high levels of stress hormones (e.g. glucocorticoids) that could only otherwise be achieved *in vivo* through exposure of mice to very stressful stimuli (e.g. restraint, exposure to predator odors such as coyote, bobcat urine or tape- recorded calls of owls). This technique will therefore reduce animal suffering, as follicles or cumulus- oocyte complexes will be collected from dead animals that themselves do not need to be exposed to stressful stimuli.

To reduce the number of animals we usually perform pilot work in cells growing in a dish, to help the design of the *in vivo* work, and also use cellular models to complete, and complement, the *in vivo* experimental work. The design of our experiments and data preparation/publications are guided by the NC3R's PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) and ARRIVE (Animal Research: Reporting of In Vivo Experiment) guidelines, respectively.

We write comprehensive Study Plans for each experiment including: a statement of the objective(s); a description of the experiments, covering topics such as proposed treatments, the size of the experiment (number of groups, number of animals in each group), and the experimental materials; and an outline of the method of analysis of the results are included (which may include a sketch of the analysis of variance and some account of the tests of significance to be made and the treatment differences that are to be estimated).

Refinement



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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use mice at all stages of development, and these include genetically altered animals and wild-types. We will use standard and state-of-the-art methods to produce genetically altered animals, alongside with specific methods to study resource allocation during pregnancy and lactation, and growth and metabolism in postnatal life. The vast majority of the procedures only incur transient pain and discomfort to the animal, and overall most of the protocols fall under the 'mild' severity level. A number of procedures we intend to do are 'terminal' (for example, euglycemic clamps, which requires maintaining a high insulin level in mouse blood for a specific amount of time, is a way to quantify how sensitive specific organs are to insulin actions, and placental transfer assays, which is applied to follow how well nutrients are transported from mother to the fetus, through the placenta) and the ones that are not, do cause the least pain, suffering, distress or lasting harm to the animals.

However, some of our proposed genetic manipulations are likely to be associated with lethality around birth, (a common observation in mice with severe growth problems in the womb) and other developmental phenotypes (e.g. anemia, which is low level of oxygen carried by red blood cells, and increased risk for malformations of the brain). It will be difficult to predict the nature of developmental phenotypes that will arise in newly generated REDACTED. However, we intend, for some selected cases, to study the molecular mechanisms that lead to those potentially harmful phenotypes. Once the phenotype is fully characterized and tissues are collected from those diseased animals, any subsequent work will be done at early stages of disease, therefore prior to the beginning of any overt discomfort.

We will minimise suffering by using analgesia (which is medication that is given to relieve pain) and/or anaesthetic (meaning loss of sensation, which may include inducing sleep) or any potentially painful procedures and by careful monitoring of the animals to ensure they are not in discomfort. Once we have established the level of discomfort that might be caused by specific genetic mutations, we will apply clearly defined endpoint criteria to minimize suffering. In addition, we will endeavour to identify markers at the cellular level that are indicative of early onset of disease, thus minimising the need to maintain mice with overt disease.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We have not considered the use of less sentient animals (sentient means ability to perceive or feel things) because our work is obligatorily focused on mammalian species, and all mammals are sentient). To achieve our objectives we need to use a mammalian species that have a placenta and that can be manipulated genetically.

Mice are the species of choice because specific genes can be manipulated (e.g. knocked-out) in either specific organs or the whole body (and importantly, the knockout mutation is



passed on from generation to generation). This allow us to understand what these genes do during development of the animal – in our case, we are interested in genes that control resource allocation (e.g. nutrients) and the roles they may play, for example, in the placenta (the organ that provides nutrients from the mother). We have ways of following up the nutrients going from the mother to the fetus, as a terminal procedure, and if a gene is very important in this process we should be able to identify it by studying a mouse that does not have that gene working properly in the placenta. We have a number of procedures in which an animal has been terminally anaesthetised, and we use analgesics and/or anaesthesia to minimize pain of some of our procedures. We use non-invasive techniques as much as we can (for example to measure blood flow from the mother to the fetus and from the fetus to the placenta, or to measure body fat composition). To investigate how stress before conception can influence offspring characteristics, we will take eggs (oocytes) from the ovaries of dead mice and expose them to high levels of stress hormones; we could not naturally achieve these high levels of stress hormones in live mice without causing significant stress (e.g. restraint, exposure to a predator).

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

To improve the quality of life of the animals we:

reduce contingent harm by group housing where possible to keep singly housed mice to a minimum. This in turn reduces any stress and stereotypical behaviour.

use environmental enrichment EE, within what is available to us at our animal facility. In general, EE is an animal housing technique composed of increased space, physical activity, and social interactions, which in turn increases sensory, cognitive, motor, and social stimulation. Igloos, running wheels, saucer wheels, fun tunnels, and other objects in the housing environment foster this sensory cognitive, social, and motor stimulation by promoting exploration and interaction. EE can be maintained through restraining (e.g.handling tunnels), thus minimizing stress when for example an injection is needed.

keep animal transportation to a minimum (most of our mice are bred in house and kept in one facility).

use analgesics to lessen pain.

provide 'behavioral' training to mice undergoing specific procedures (e.g. blood pressure measurements by tail cuff; acclimatization to hypoxic chambers; acclimatization to metabolic cages)

use scoring sheets to monitor the health of animals undergoing procedures.

use mash, with improved nutritional composition/palatability, for animals likely to lose weight- e.g. before they start a treatment where weight loss is expected , and kept for the duration of the procedure.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Laboratory Animal Science Association (LASA) guiding principles documents of aseptic technique (https://www.ubs.admin.cam.ac.uk/files/lasa_aseptic_surg.pdf)



ARRIVE (Animal Research: Reporting of In Vivo Experiment) guidelines for preparing papers for publication (<https://www.nc3rs.org.uk/arrive-guidelines>)

PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning our experiments (15 topics including formulation of the study, dialogue between scientists and the animal facility, and methods) (<https://www.ncbi.nlm.nih.gov/pubmed/28771074>).

British Journal of Cancer (2010) May 25;102(11):1555-77
(<https://www.ncbi.nlm.nih.gov/pubmed/20502460>)

How will you ensure you continue to use the most refined methods during the lifetime of this project?

To be informed about latest advances we will primarily use the National Centre for the Replacement and Reduction of Animals in Research (NC3R) website (<https://www.nc3rs.org.uk>). It provides an extensive library of 3Rs guidelines, resources, practical information and themed hubs. It also provides links to publications, other online resources, and video and training materials.

The REDACTED Biomedical Services website provides a portal to numerous sources of information, with a few listed below:

(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 <https://www.nc3rs.org.uk/welfare-assessment>
<http://enrichmentrecord.com/>

<https://science.rspca.org.uk/sciencegroup/researchanimals/implementing3rs/rodentwelfaregroup>

Implementation of the advances will be defined on a case-by-case basis, and will be informed by the latest NC3R recommendations.

Explain the choice of species and the related life stages

In species like humans, wellbeing and health in later life is in part determined by past experiences whilst in the womb. Life in the womb in mammals is made possible through the action of a specialized organ, the placenta, that provides the maternal nutrients and gases necessary for the growth of the offspring. We know very little about the communication between the fetus, the placenta and the mother and the 'signals' that regulate this seamless cooperation. For our studies we need to use a placental mammal, with placental characteristics close to human ones, and whose genome can be edited with ease so that we can interrogate the role of genes in the signalling between the placenta, the fetus and the mother. The mouse is the only animal that can fulfil this criteria, and thus the obligatory model for our studies. We will use genetically altered mice and also wild-type mice for our studies.

We will use mice that are pregnant and breast feeding so that we can study how genes can influence the nutrient resource allocation, through the placenta and the maternal milk. We will also study adult mice to understand better how life in the womb determines



wellbeing and health in later life. When choosing both the animal species and life stages for our work one other overriding factor was the possibility of modelling human disease (e.g pregnancy complications and disease in later life, such as diabetes and heart disease), so that the data obtained could be applied to clinical research. Moreover, we made the conscious decision of using models and life stages that were amenable to therapy (drugs or nutrient manipulations) with the view of preventing, delaying or curing pregnancy complication and metabolic disease.



59. Identifying genetic and biological factors and treating Otitis Media in mouse models

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

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 is the aim of this project?

To identify means to treat otitis media by determining the genetic basis, pathobiology and role of infectious agents in otitis media using mouse models of conductive hearing loss.

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

Why is it important to undertake this work?

Otitis media (OM) is an inflammatory disease of the middle ear that is extremely common in children. Chronic OM with effusion (COME), also known as glue ear, is the most common cause of hearing impairment potentially causing learning delays and behavioural problems. The preferred treatment for COME is the insertion of grommets (the most common surgical operation carried out in the UK) but the effectiveness of this procedure is relatively low. Acute OM (AOM) associated with bacterial infection is the commonest reason for antibiotic prescription in the UK. There are currently no other effective



treatments for COME or AOM. This significant clinical need can be met through discovering biological factors that influence OM using mouse models, these can then be tested as potential targets and means to better treat or prevent the disease.

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

By exploring the biology of the infected middle ear in mouse models of otitis media we will gain a fuller understanding of the genes and associated pathways that predispose to the disease, this data is likely relevant to disease in human. These combined studies will provide knowledge on host pathways, molecules and cells that can then be tested as candidates for improved therapy to better treat (drugs) or prevent (vaccines) disease.

All data obtained during these studies will be made publicly available through peer-reviewed publication and presentation at local, national and international scientific meetings and conferences.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The data realized in these studies will inform us on factors relevant to human OM disease that will then be tested to potentially offer better ways to treat the disease in the clinic.

We estimate that we will study genes and pathways in two to four novel REDACTED over the time course of this licence; preliminary studies of the OM phenotype and host microbial interaction would take up to 2 years investigation in each case. Further detailed investigation of host-microbial interaction using human otopathogens will be undertaken in at least two established or novel REDACTED; in each case these studies would last for up to the duration of this licence and include investigations for therapeutic intervention (estimated 2 routes of vaccine administration of up to four formulations and 2 routes for drug administration of different immune modulating and antimicrobial substances). We would seek an industrial partner to take forward any promising novel treatment regimen once found.

How will you maximise the outputs of your work?

Through coordinated studies with an existing set of local, national and international collaborators and clinical partners we can enhance the level and diversity of output from our studies in the mouse models of OM. To take forward any promising novel OM treatment identified, we would seek an appropriate industrial partner.

Mouse models will be made available to other research groups and all data obtained and protocols used in our studies will be made available by publishing it in free to access format in high quality journals following peer review.

Output from the studies will also be presented to the scientific community through presentation at local, national and international meetings and conferences (including the biennial International Society for Otitis Media conference).

Species and numbers of animals expected to be used

- Mice: 31,100 mice



Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The most common experiment is the infection of mice by bacteria, typically these bacteria are applied through the nose of the mouse and then the bacteria in the nose and ears of the mice are assessed after 1 to 7 days of infection when the mice are killed humanely. Blood and other fluids are collected to assess the effect of the interaction between the bacteria and the mouse. In some experiments changes in the middle ear are monitored after administering substances that might improve the disease outcome.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

There is very little evidence of pain, distress or suffering through the development of OM for the animals used in our studies. Our experiments are carefully planned and implemented and any new substances to be administered are first tested in only a few mice. All mice used in the study are carefully monitored by the person carrying out any procedure and welfare assessment is recorded throughout the lifetime of the animal, these practices are well established in our animal house.

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

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

Over 80% of mice should be subject to only sub-threshold severity and the remainder would experience a maximum of Moderate severity during their lifetime.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 auditory system is complex and it is not possible to perform meaningful studies on hearing without using the intact animal. The middle ear itself is a complex structure that communicates with the nasal passages and nasopharynx via the Eustachian tube. Otitis media is inflammation of the middle ear space and is characterised by the accumulation of a complex inflammatory fluid and hearing loss, again this cannot be properly reproduced



outside the animal. The natural route of middle ear infection is by ascending infection of the Eustachian tube, a feature that we mirror in our studies. To study the mechanisms of otitis media and treatment it is therefore necessary to work with living mice.

The mouse shares similar anatomy and physiology of auditory system and also shares disease genes that predispose to otitis media in humans. The mouse genome is known and genetic manipulation is possible and reagents are readily available to enable meaningful cellular, biochemical and immunological studies to be carried out. The hearing organs in lower animals such as frogs and fish are anatomically different from mammals and otitis media cannot be modelled in these species or in flies.

What was your strategy for searching for non-animal alternatives?

Some *in vitro* experiments utilising specific types of host-derived cell (e.g. stem cell derived immune cells or middle ear derived epithelial cells) can be used to examine certain features of that cell type and its role in host-microbial interaction and signalling.

Why were they not suitable?

The complex inflamed middle ear environment, which is the key feature of otitis media, cannot be recapitulated *in vitro*.

There are no effective alternatives to using live animals when carrying out studies on hearing and otitis media.

Reduction

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

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

Based on extensive experience gathered for these types of study over many years of breeding and experiment and appropriate statistical calculations for the number of animals required we design robust experiments that minimize the number of animals necessary for study.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

For otopathogen infection studies we derive spontaneous streptomycin resistant versions of the strain before experiment whenever possible. This enables us to plate mouse samples on antibiotic containing media and avoid the loss of approximately 19% of data from count plates that occurs due to overgrowth of mouse indigenous flora when using non-antibiotic culture plates.

Power calculations used to decide the desired number of animals for experiment are carried out in consultation with a local statistician.



What other measures apart from good experimental design will you use to minimise numbers?

By carrying out several different analyses from multiple tissues and fluid obtained from a single mouse we have maximized the information that we obtain from any single animal and reduced the overall number of animals required for experiment. We also take bone marrow cells and middle ear cells from mice to grow in the laboratory, by studying these cells we gather relevant information on their function and potential role in OM disease. Some studies to explore cell function are also carried out in the laboratory using available cell lines.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The mouse will be used in the studies associated with this licence. OM occurs spontaneously in these mice and there appears to be little pain, suffering, distress or long lasting harm associated with this phenotype. In order to investigate the disease, we use minimally invasive procedures and anaesthesia wherever possible. Mice are often inoculated with pathogens that are placed in the nasal cavity of the mouse and/or treated with medicines to assess their potential for improving hearing and disease. Adverse reactions to these tests are rarely seen but may include some unforeseen response to particular medicines or vaccination.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The auditory system is complex but the architecture and function of the mouse ear is very similar to that of human. The mouse is the lowest mammalian species in which the full range of studies necessary for the scientific investigation of otitis media can be effectively carried out, with findings that are relevant to human disease. Some experiments on hearing can be carried out on frogs and fish but these are not informative on human OM as the animals do not get the disease.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We continually assess the impact that the experiments carried out have on the animals used. Welfare assessment is a key aspect of this project and will be performed and recorded throughout the lifetime of the mice, these practices are well established by the highly trained and experienced staff at our animal facility, where all mice are bred and housed. In addition, where ever possible non-invasive tests only causing temporary discomfort will be performed.



What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The NC3Rs and ARRIVE guidelines will provide the best practice guidance for the use of animals in our procedures.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Our Institute sends out regular updates on the 3Rs and once a month I check the website for any new information that is relevant to our studies. Through continual monitoring of the scientific literature and by attending relevant national and international meetings we can keep abreast of any advances in the scientific field that could help reduce the number of animals used for study or lessen the severity within any given experiment.

Explain the choice of species and the related life stages

We investigate otitis media, a very common disease of human, that results in hearing loss. The mouse has similar anatomy and physiology of the auditory system compared to human and OM in the mouse closely resembles the disease in human. Mutant REDACTED develop symptoms of OM spontaneously in adults at about 4 to 5 weeks of age. The mouse is the lowest mammalian species in which the effective scientific investigation of OM can be carried out.

Many *in vivo* animal otitis media infection and treatment studies are carried out in the chinchilla (*Chinchilla lanigera*) because its auditory system is similar to that of man and disease can be reliably induced through direct inoculation of the middle ear. However, natural translocation of bacteria to the ear from the nasopharynx is difficult to achieve in this species and limited reagents are available for biochemical and immunological analyses.





60. Immune phenotype and dynamics in ocular autoimmune disease

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Animal types	Life stages
Mice	juvenile, adult, 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 is the aim of this project?

To identify and measure mechanisms that regulate ocular autoimmunity. To assess the efficacy of therapeutic manipulation of some of these mechanisms.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 known that in some diseases the immune system attacks otherwise normal tissue with potentially devastating consequences. Many human illnesses are autoimmune in origin and one is idiopathic inflammatory eye disease (generally known as uveitis). The chronic nature and cost of treatment for these conditions places an enormous burden on modern health care. Uveitis is a leading cause of blindness in working age adults living in developed countries; new cases present at a rate of 75 people per 100,000 and up to 35% of patients become visually disabled. Poor vision secondary to uveitis impacts significantly on general quality of life. The minimum cost of treating one patient with uveitis for one year



in Europe in 2008 was £3,600. The current state of knowledge is that autoimmune diseases arise in the context of a permissive genetic background, following a triggering event. Following this triggering, the disease process can be characterised as a chronic inflammation, that damages normal tissue function and that waxes and wanes with time. Many different cell types are recruited to the target organ, showing that autoimmunity arises through the co-ordinated effects of many different signalling events. The complexity of the response is the reason that studies of the whole organism are essential for research into treatments for autoimmune disease. This research into animal models of uveitis will have the benefit of revealing how specific mechanisms can be manipulated to produce an improved clinical outcome.

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

New information: We expect to develop new information characterising the dynamics of ocular disease. We will also produce new information on the regulation of tissue health in ocular inflammation by specific molecules in signalling and effector pathways. Finally, we expect to have shown the impact of novel interventions.

Publication by poster and oral presentation at national and international meetings such as the annual meeting of the association for research in vision and ophthalmology (ARVO).

Publications in peer reviewed journals such as the Journal of Immunology or Nature Immunology. We are also active in outreach to patient groups and lay members of the public, where we present and discuss research.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Short-term benefits (throughout project): Increase in knowledge of uveitis, and the development of models and strategies for analysis that can be applied to human disease. Training of individuals who specialise in ocular research.

Medium-term (over 2-5 years): Communication to a professional audience through publication and a wider audience through outreach to patient and public groups.

Long-term (greater than 5 years): Validation of therapies for the treatment of uveitis in humans and animals using novel approaches based in part on the understanding of the disease developed during this project.

How will you maximise the outputs of your work?

We maximise the outputs by seeking out collaborations with scientists working in other disciplines (engineering, mathematics) who can use data produced in our investigations to develop novel approaches to mathematical modelling and image processing.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms



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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The project studies ocular inflammation at the back of the eye. This is an autoimmune process with minimal systemic side effects. It arises following treatment that leads to an immune response that is directed at the retina and causes inflammation, and tissue damage. This treatment requires the injection of substances that initiate the immune response, or of cells that cause the disease. Following the induction of disease, images from the back of the eye can be recorded with specialised equipment, to be analysed to determine, for example, if an experimental treatment can reduce disease. This sometimes requires that animals receive a general anaesthetic. Rarely (1% of experiments) following whole body irradiation, animals will receive a bone-marrow transplant. This is a stressful procedure of moderate severity.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We carry out these studies in mice, for whom sight is a secondary sense. In general, uveitis is not painful and animals show little abnormal behaviour during the course of disease. As in humans, the initial disease sets up a long-standing grumbling inflammation that comes and goes through time. By monitoring this in the long-term we can investigate how patients with chronic persistent disease might be treated. Generally experiments are carried out over a period of months, but some studies last as long as a year. Animals that receive a bone-marrow transplant recover to normal behaviour over about a week.

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

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

Mild and subthreshold in 99% of cases. In 1%, where animals receive bone-marrow transplants, the severity is moderate because of this.

What will happen to the animals at the end of the study?

- Used in other projects

Replacement

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

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

The study of fundamental and novel mechanisms of disease continues to rely on whole animal models because the complexity of the pathology cannot be mimicked in simpler systems. All modern drugs developed for the treatment of human disease have relied on



the use of animal models. Experimental autoimmune uveitis (EAU) in mice is a mild disease that does not produce systemic distress related to the disease process and uveitis cannot be studied in simpler models, as the effects on the anatomy of the eye are critical in assessing the disease. The rodent model is therefore the least severe approach to addressing our objectives. Suffering is minimised by using the least severe experimental model relevant to the underlying mechanism and using clinical monitoring that detects the effects of interventions at early timepoints.

What was your strategy for searching for non-animal alternatives?

Where appropriate we use in vitro models of immortalised cells and primary cell lines to assess the biochemistry of individual pathways, identify appropriate drug targets and validate them prior to use in more complex disease models.

Why were they not suitable?

In vitro models do not recapitulate the complex anatomy of the eye or the physiology of the disease process of EAU, nor are they suitable for studying how the disease develops through time, which is a crucial aspect of these chronic conditions in both animals and 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?

Breeding is estimated at 500 animals per year, based on current usage in our group. Experimental usage is estimated at 300 per year which is 10 experiments per year divided between two post-graduate/post-doctoral researchers.

Sample size calculations are calculated by computer modelling to estimate how many animals are necessary for an experiment to have approximately an 80% probability of detecting a real difference between groups.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Online tools were consulted but by and large they assume the use of parametric statistics, which are inappropriate for clinical assessment of EAU and assessment of EAU by cell counting, even after standard normalisation procedures. We therefore used statistical packages in R that allowed us to estimate sample sizes.

By comparing cell numbers in treated versus untreated animals, we can use a stepped design, where the total numbers for the experiment are split over two or more time points, coupled to a Bayesian analysis, so that if we see larger effects than we have modelled for, we can stop the experiment when a robust data has been obtained.



What other measures apart from good experimental design will you use to minimise numbers?

We use efficient breeding, pilot studies, computer modelling. Tissue sharing occurs on an ad hoc basis.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

EAU is a model of autoimmune disease that has little impact on mouse behaviour, but offers a powerful model to study the basic mechanisms of the disease and the effects of interventions on immune system function. In comparison to other disease models, the levels of distress are lower and this allows for longer term experiments which can address important aspects of the disease process.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Autoimmunity is an emergent property of vertebrate immune systems. It is influenced by age, sex and environment. While individual pathways can be isolated in some circumstances, the disease cannot be modelled in a less sentient species.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We continue to refine our methods for monitoring clinical disease and are actively studying unsupervised algorithm and machine-learning based approaches to allow us to analyse disease through time, develop more sensitive measures of treatment efficacy and correlative approaches to better quantify immune changes.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We participate actively in 3Rs seminars, presenting our approaches and learning from others.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Through continuing education including attending and contributing to 3Rs focused seminars and lectures.

Explain the choice of species and the related life stages



We use animals that are known to be susceptible to the disease experimental autoimmune uveitis (EAU), and which develop this disease from the juvenile (4 weeks of age) stage onwards. Typically mice are 6-8 weeks of age at the time of experiments.





61. Immune responses to persistent infection and vaccines

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The project aims to understand how the immune system responds to viruses, cancers and vaccines, and to use this information to design better therapies and new vaccines.

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

Why is it important to undertake this work?

Infections and cancers are major human health threats. For infections we know that immune responses are important and we also know that vaccines are one of the most effective medical interventions ever created. We still lack vaccines for many important infections such as hepatitis C, HIV, Cytomegalovirus and Epstein Barr Virus. In recent years the power of the immune system to protect against cancers has been revealed. The same processes that generate effective immune responses against viruses can also impact on cancers. The aim of the project is to unpick in detail how the immune system



recognises viruses and vaccines and to harness this information to produce better interventions in a range of settings.

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

New information that will help to understand the nature of the immune responses to viruses and vaccines, the role of specific new types of immune cell and also how we may harness these responses to tackle cancer. The information on how best to use the vaccines can be directly used to design better strategies (types of vaccine or combinations of vaccine) in different infections and cancers. We aim to publish the results in peer-reviewed journals and conferences.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Within the timescale of the project we hope to have answered a few key questions about how the immune system responds to vaccines and viruses. For example the role of a new type of immune cell (the REDACTED cell) and related cells that have not been well explored in this context. We also hope to have answered questions relevant to the use of such vaccines in cancer therapy to help design trials in patients for cancers such as melanoma. These trials and vaccines are being designed over the next 2-3 years and our results can help contribute to making the best vaccines from the different choices available. The research community will also benefit from knowledge obtained.

How will you maximise the outputs of your work?

We have collaborated widely to improve the outputs of the work and have colleagues in labs across the world whom we will share our data with. This includes academic colleagues in universities as well as collaborators in biotech companies aiming to develop vaccine strategies. We also will present the data at scientific conferences in the fields of immunology and infection as well as cancer immunotherapy and publish the data in respected journals (recent publications are in Cell Reports and Nature Communications for example). The data published will include the relevant negative data as well as positive results which can be followed up by the field.

Species and numbers of animals expected to be used

- Mice: 15000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically a mouse will receive an injection (eg. immunisation with adenovirus) followed by four blood tests taken over a period of eight weeks to test the effect of the immunisation. A



proportion of these animals may also be challenged with an infectious organism (e.g. influenza) or administered with an anti-cancer compound after a challenge with a tumour.

All animals will then be killed humanely before one year of age.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

There will be minimal impact from the immunisation and at most short term general effects related to any vaccination (<48 hrs). There may be some transient pain associated with a subsequent blood test to assess immunogenicity.

For experiments where antibodies will be injected (eg: depletion of specific cell-subsets using monoclonal antibodies), there is a risk of reaction against the 2nd dose of the antibody (e.g. a severe allergy). Should this reaction occur (recognised by rapid onset of immobility and a change in breathing pattern) mice will be killed immediately.

For experiments involving tumour challenges we will monitor the size of the tumour over subsequent days to weeks to assess growth and stop the experiment appropriately according to well-defined humane endpoints (in particular tumour size).

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

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

Expected severity for protocols 1 and 2 are expected to be 50% moderate based on previous work. Mice in protocol 3 will mostly experience sub-threshold severity and it is estimated that less than 5% will experience mild severity from ear notching or blood sampling.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Despite efforts to carry out replacement strategies using organ cultures, not all of the cell types involved in vivo can be successfully grown in vitro and therefore the immune responses cannot yet be fully replicated in vitro. Consequently, animal models are still required to study the mechanisms of immune responses during vaccination and infection.

What was your strategy for searching for non-animal alternatives?



We are developing models to study some of the simplest elements of virus persistence in cell culture. For example we have set up infectious model systems for HBV and HCV where intracellular responses can be assessed. We are also working on models of human tonsil where some aspects of immune homing can be measured in vitro. If we have indications from these cell culture models which could lead to replacement of the planned in vivo work we would take advantage of this.

The lab works with human studies including analysis of Phase 1 clinical trials. The experiments planned are only those where we cannot achieve the same objectives by analysis of human subjects.

These trials have focused on the development of adenoviral vectors for prevention and therapy of Hepatitis C Virus and for protection against Respiratory Syncytial Virus (RSV). We have ongoing studies of HCV, HBV, HIV and CMV in the lab, as well as analysis of human innate T cell responses such as REDACTED cells which can control bacterial and viral infections in tissues. These data have been used to plan better experiments in the mouse model, which can then be used to address questions most relevant to the clinical setting.

Why were they not suitable?

Such ex vivo experiments cannot address the immunogenicity of a range of vaccines - we can only test very few vaccines in humans. To define which of the potential approaches might be best (and safest) we still require an intact and flexible in vivo system. It also needs such a system to assess the efficacy of induced responses against challenge. Finally, we cannot fully address the mechanism of action of specific components of the system (eg: specific cell types) unless we use an in vivo model where they are lacking or modified.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 largely estimated them based on previous usage over a period of time. For each individual experiment the numbers used are based on power calculations for the appropriate endpoint (e.g. immunogenicity or protection) - these numbers are typically in the order of 5-10 per group for most experiments. The total numbers included also take into account mice needed to establish breeding colonies for specific experiments, although clearly we aim to keep those numbers to a minimum.

During the analyses of immune responses in the key experimental protocols, we typically find small standard deviations (measures of how variable the readings are). In these experiments we are also looking for large effects (eg: 5 fold), but also those which distribute over time, which increases the power of detection. In a typical experiment, if the vaccine induced T cell response is 2%, with a standard deviation of 0.3, we can detect a 50% reduction in response in the experimental group (with similar standard deviation), with $p=0.001$ (a p value less than 0.05 is generally regarded as significant in such studies). If



the standard deviation were increased to 0.66 (range 1.2-2.8 for the control group and 0.2-1.8 in the experimental group), we can still detect a response at $p < 0.05$. Thus for experiments of this kind 5 per group provides appropriate sensitivity to detect moderate effects even at a single time point.

For gene expression experiments $n=3$ is the minimum required to generate statistically robust datasets and we have already achieved this. We have a bioinformatician working in the group who can provide further ongoing advice about statistics. Using group sizes of 3 we can use standard statistical tools (based in the package R) to define gene expression profiles taking into account multiple comparisons using RNAseq techniques. For the emerging technology of single cell RNAseq, which gives greater resolution of transcriptional activity, we have developed parallel techniques which also work on groups sizes with a minimum of 3.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

In many cases, the numbers of animals required will be reduced by repeated, longitudinal measurement of responses through serial blood analysis. The immune response to an immunisation may be measured weekly in a set of five mice over a period of several weeks rather than culling five mice every week. This approach also provides valuable information on the development, or not, of protective immune responses prior to challenge with a pathogen or tumour. We will be using the NC3Rs' Experimental design assistant which we have used during our previous licence.

What other measures apart from good experimental design will you use to minimise numbers?

Animal numbers will be optimised by purchasing wild type mice for specific experiments rather than breeding them. Genetically modified strains bred in-house will be closely managed and where possible both males and females used.

We routinely use many tissues from a single animal at the end of the experiment instead of using individual animals for each tissue. This overall reduces the number of animals required.

Occasionally we will share tissue with other members of the group to perform genetic analysis of responses.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The complex interaction of immune cells and tissues dictates the need for using model organisms. Mice have a relatively short lifespan and are also considered the least sentient



species available for this type of analysis of immune correlates of protection for many human pathogens.

The immune system of mice has been well-studied over many decades and there are well-optimised reagents and protocols available. The immune cell biology of mice currently provides the best and most highly characterised model for understanding human immunity.

We are able to use the wealth of published data to design and refine our experiments leading to contribution to this knowledge through our own publications. This contribution will in turn reduce and refine animal experiments in this field.

In the previous licence we used rats to test models of hepatitis C and the vaccines against this disease. This was successful but we can refine the experiments now using mice as a new model has been developed using the same virus. This will provide an important refinement for this study.

When experiments involving *Listeria* challenges are undertaken we will use *Listeria monocytogenes* DPL 1942 expressing ovalbumin when possible. It is genetically attenuated and therefore less pathogenic than wild type *L.monocytogenes*.

Animal welfare and well being will be closely monitored in close collaboration with the highly trained BMS staff.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The mouse immune system has been shown over time to be a good model to use for human immunity, especially in the context of vaccines. Only mammalian immune systems contain all the relevant components we need to analyse and the well-defined structural composition (lymph nodes and organs) makes this a very accurate system to define the totality of the immune response of the animal. The experiments require days or weeks for the immune responses to develop and require an intact adult animal for completion.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The mouse is a very well defined model for immunologic experiments and the model proposed using MCMV, adenovirus vectors and other viruses is highly reproducible and therefore can be used more efficiently and with the maximum refinement. For example we have developed the use of the non-replicating adenovirus model, which has less impact on the animal and used protocols we have refined over time. Thus we have minimised the amount of blood drawn per time-point by optimising lab immunology protocols and minimised the numbers of bleeds required per protocol. The use of a range of highly defined genetic (genetically altered) models with specific immune defects to probe the mechanisms of antigen presentation and T cell activation/homing make the mouse model especially appropriate. In particular we can now use mice with tissue-specific expression of Cre recombinase, a molecule which recognises specific (Flox) sites in DNA and allows activation of such genes in vivo. Such animals can be infected with adenoviral vectors where the antigen can be modified through insertion of Flox sites, or crossbred with specific genetically modified strains expressing genes under Flox sites: this very specific approach reduces the chance of encountering a harmful phenotype which could be encountered using genetic knockouts in all cells. We will also in some experiments use a



doxycycline or Tamoxifen-inducible Cre system to allow for temporal control of Cre expression, allowing us to refine the model further.

Following an amendment to our previous licence we developed a challenge model for Hepatitis C in rats. A mouse-adapted strain of Rat Hepacivirus (NrHV) is now available meaning we are now able to develop this model in mice.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The NC3Rs website provides guidance to best practice through e-learning, blogs and videos.

Information to aid design and planning of experiments is also available, in particular, the experimental design assistant.

Guidelines for the welfare and use of animals in cancer research by Workman, P. British Journal of Cancer (2010) 102(11), 1555-77) provides guidelines for the tumour work under protocol 2.

Relevant LASA publications https://www.lasa.co.uk/current_publications/

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The REDACTED has an NC3Rs regional program manager and can provide expert advice and co-ordinating the sharing of best practice. There are also termly meetings for all PPL and PIL holders where information on the 3Rs is often disseminated.

We also use the handbook, Festing MFW and Altman DG, 2002 ILAR J; Festing MFW, Overend P, Gaines Das R, Cortina Borja M and Berdoy M, The Design of Animal Experiments (2002) Laboratory Animals Ltd., London, to inform experimental design.

Explain the choice of species and the related life stages

Adult mice will be used in this project. There are well defined models and reagents are readily available.





62. Immunity and haematopoiesis

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The aim is to identify novel pathways that regulate immunity and haematopoiesis during homeostasis 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?

The immune system has evolved to protect the host and fight disease. It works throughout the body, interacting with the multitude of organs and tissues, and the nervous system, to maintain immune homeostasis and tissue repair, and defend from infection. However, there are occasions when the host immune system fails to combat the attack.



For example the World Health Organisation (WHO) estimates that virally and bacterially-induced Pneumonia and diarrhoea combined lead to 29% of all child deaths globally; that over 880 million children require treatment for helminth parasites; and that in 2017 there were 219 million cases of malaria (a protozoal parasite) worldwide with 435,000 deaths. By understanding the immunobiology of infection we aim to discover new opportunities for better prevention and treatment of disease, to reduce human suffering. For instance, one of the greatest achievements of the 20th Century was the almost total eradication of poliomyelitis following the development of the polio vaccine (99% reduction from 1988 to 2010). However, for many diseases we still have no working vaccine.

In other instances the immune system over-reacts leading to auto-immunity such as inflammatory bowel disease, or may be inappropriately elicited e.g. when pollen or house dust mites cause allergy and asthma. In many of these inflammatory diseases the immune system becomes dysregulated and anti-inflammatory responses that should protect from excessive collateral damage become misdirected. Asthma is a common chronic inflammatory disorder that is characterised by inflammation and hyperreactivity of the airways, and afflicts approximately 235 million people worldwide including 1 in 5 children in the UK. Significantly, 5 – 20% of sufferers develop severe asthma that is not controlled even by newly developed treatments (arising from immunology research). Furthermore, there is growing awareness that acute asthma exacerbations are often associated with viral infection. WHO also predicts that by 2030 chronic obstructive pulmonary disease (COPD), for which there is no effective treatment, will become the third leading cause of death globally. It is critical that we understand the fundamental regulation of the immune system so that we can continue to develop new therapies to relieve human suffering.

Notably, in some circumstances the cellular and molecular peacekeepers of the immune system can be silenced and fail to destroy abnormalities like cancer. It has been estimated that in 2018 ~9.6 million people died from cancer (WHO). However, recent studies of the immune system, relying heavily on fundamental experiments using mice, have led to the development of new therapeutics that prevent immune suppression and enhance immune-mediated killing of cancer cells. Whilst, this is a significant breakthrough in cancer treatment, and yet another validation of the importance of studying the immune system, not all cancers can be treated successfully with these new drugs, highlighting the importance of continued work to discover new targets.

Interestingly, the maintenance of immune balance impacts on tissue homeostasis. For example, a lean healthy body is associated with the presence of a type-2 immune microenvironment, whilst obesity is associated with type-1 immune reactions. The incidence of obesity is increasing (1 in 4 adults, and 1 in 5 children in UK are now considered obese - NHS), with significant effects on susceptibility to other diseases including cancer and asthma. Once again, it is critical that we investigate the contribution of the immune system to the deleterious effects of the over-consumption of food.

The aim of this project is to investigate the fundamental regulation of immune responses to identify new targets for therapeutic intervention.

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

The immune system is critical for the defence of the body from infection, but it can also misfire and start to attack the body (autoimmunity). Infectious disease is the biggest killer of mankind world-wide. Viruses alone kill more than twice as many people each year as cancer. Asthma is a common chronic inflammatory disorder that is characterised by inflammation and hyperreactivity of the airways, and afflicts approximately 235 million



people worldwide. Although many asthma patients respond to corticosteroid medication, such treatment can be associated with negative side-effects. Significantly, 5 – 20% of asthmatics develop severe asthma that is not controlled by standard treatments, and they account for a significant proportion of the morbidity, mortality and cost of the disease.

There is therefore a desperate need to understand how we can selectively arm the immune response to combat pathogens, or pacify inappropriate immune reactions that harm the host. With this knowledge we can work towards better prevention of disease (through vaccination, prophylaxis and good practice), improvement in diagnosis at an early stage to increase the benefit of treatment and/or reduce transmission, and development of new therapeutics. These are long-term goals, but the objectives of this project are to provide some of the necessary information to make this possible.

Consequently, the immediate core benefit of the work will be that we will increase our fundamental understanding of the molecular and cellular pathways that regulate haematopoiesis (the process of making immune cells) and immunity, especially asthma and allergy. A better understanding of these mechanisms and specific candidate genes will allow us, and the scientific community, to develop novel approaches to modulate immunity and blood formation to prevent disease.

We have disseminated our research in over 90 manuscripts published over the past ~9 years. Furthermore, within a similar time-scale we have produced two novel antibodies (anti-IL-25 and IL-25 receptor) that have been humanised and licenced to pharmaceutical companies, for the treatment of asthma and allergy.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The important long-term benefit of our research is to contribute to the development of treatments to both prevent and treat immune and haematopoietic diseases. We have already identified new therapeutic targets and developed drugs that are in preclinical development for the treatment of asthma and allergy and continuing with these studies will enable us to support work to take these drugs forward for clinical trials. In addition, we will continue to pursue the identification of new treatment strategies, for example by using disease models to identify novel gene candidates that can be targeted therapeutically.

In the longer-term we expect our findings to inform and guide new therapies in a variety of human diseases including asthma, allergy, autoimmunity and obesity.

The generation of transgenic animals within this programme will also be valuable to other scientists aiming to develop therapeutics to these and related diseases by facilitating more rapid progress in their investigations.

How will you maximise the outputs of your work?

It is important that our work is made available to the wider community once we are satisfied that it is robust and reproducible. All our published research manuscripts are available through Open Access.

I am also invited to present our work at national and international conferences where we can present work in-progress before publication. This can generate new ideas and lead to



collaborations. Indeed, we have a number of collaborations world-wide, with research scientists and clinicians, in which we provide know-how or reagents.

Species and numbers of animals expected to be used

- Mice: 70,000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Our experiments in mice will mimic, in an experimentally controlled fashion, the various scenarios the immune system has to deal with (e.g. vaccination, infection, autoimmune disease, asthma, inflammation, cancer). The majority of mice are expected to experience only very mild, if any signs of discomfort.

We will administer substances by a variety of routes, choosing the most suitable and least invasive route possible e.g. most commonly by injection into the peritoneal cavity, in the water or food, via inhalation through the nose, or by injection into the blood.

Some mice may undergo surgery (e.g. to enable injection of cells into the kidney capsule). Surgery may also be performed to investigate how the nervous system interacts with the immune system.

Most experiments are completed within 2 to 4 weeks. For instance to model asthma, ragweed pollen extract is administered through the nose for five to ten continuous days, or cytokine may be given by three doses into the peritoneum. Two blood samples may be taken to monitor potential improvements in asthma symptoms. The lung function of mice may then be assessed by plethysmography (a procedure ease of measuring breathing, performed under terminal anaesthetic).

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The majority of mice are expected to experience only very mild, if any signs of discomfort, and are regularly monitored during studies.

However, some animals will lose weight in response to infection or inflammation. If animals lose up to 20% of their body weight they will be killed humanely as soon as they are identified.

Cancer studies involve the development of tumours. For example, the onset of colorectal cancer can lead to anaemia and weight loss. Such animals are observed at least twice daily and will be killed humanely to avoid suffering should they show signs of ill-health (e.g. weight loss, deterioration in body condition).

A few animals might experience weight loss with bloody diarrhoea as a symptom of inflammatory bowel disease (IBD). We will ensure that the experimental design will keep



the number of mice that experience any form of discomfort as small as possible. Such mice will be killed humanely as soon as they are identified.

Temporary impaired mobility of one limb may be observed whilst studying how the immune system repairs damaged nerves, but this is expected to resolve within 2 - 4 weeks. Failure to resolve will result in the mouse being killed humanely.

Mice will be given pain relief prior to recovery from anaesthesia and whenever necessary to alleviate pain as advised by the veterinarian.

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

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

Approximately 80% of mice will be below the severity threshold.

Approximately 15% of mice will reach MILD severity.

Approximately 5% of mice will reach MODERATE severity.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Although we undertake many experiments using ex vivo cells, the existing in vitro assays do not accurately mirror the more complex molecular and cellular interactions that regulate immune and haematopoietic cells in vivo, leaving no practical alternatives to studies in whole animals. Despite the initiation of studies (by others) to perform computer modelling of the immune cytokine network these systems do not currently represent a viable alternative to experimental research. In particular, translational aspects of our research, such as the modulation of the immune system, are only possible in an in vivo context.

What was your strategy for searching for non-animal alternatives?

We do perform numerous in vitro assays to both define the function of the factor or cells under study, and to refine how we should continue the research so as to optimise the information gained. However, even these assays are better performed with primary cells or tissues ex vivo rather than with cell lines that may have an altered state due to their long-term culture. In this way we can better predict how the molecule will act in vivo.

We are currently investigating the potential for using tissue organoids in which innate immune reactions can be investigated, and specific haematopoiesis programmes monitored and manipulated.



We undertake human studies. With collaborators we have refined an allergen challenge model in which skin blisters can be raised at the site of immunisation to give access to serum and cellular infiltrate.

We also have a collaboration to study primary cells from asthma patients in vitro.

Why were they not suitable?

It is not possible to recreate, in vitro, the multi-cellular organisational structure that comprises the immune system. There is incredible complexity to the immune system e.g. immune cells may be produced in the bone marrow, but need to migrate to the thymus to develop and mature, before migrating again to the site of infection to combat disease, before migrating again to provide a memory of the battle so that they can be recalled rapidly if the same infection is detected (even years later). This diversity of microenvironments and time-scale cannot be reproduced in a culture dish or isolated organoid.

There are critical ethical issues that surround investigations involving human volunteers. The use of human cell culture and blister analysis offers tremendous insight, but before we attempt to manipulate the human immune system we need significant amounts of preclinical information that help to inform on the likely efficacy and potential side-effects.

Reduction

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

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

The group have attended courses on experimental design and statistical approaches and have sought further statistical advice from colleagues whenever necessary. Experimental animal numbers will be determined using available information, factorial design and power analysis to ensure that minimal numbers are used. Where new protocols with unfamiliar end-points are undertaken statistical advice will be sought to ensure group size and experimental design are sufficient to account for the expected variance. Pilot studies, will be used to test the practicality of experimental design and provide estimates of variability for power analysis to determine future sample sizes. Where appropriate, experiments will exploit a within-sample repeated-measures design to maximise the statistical analysis from fewer animals.

We have considerable experience in the generation of gene-modified mouse strains and constantly assess production success rates to ensure that we optimise and maintain standards of productivity. We are also implementing new CRISPR-based protocols aimed at reducing the numbers of mice used to produce gene-modified strains and the complexity of breeding programmes used to produce compound strains with gene-modifications at multiple alleles.

We also have extensive experience in managing complex breeding programmes. This may be to generate conditional knockout mice, requiring the intercrossing with specific Cre-expressing mouse strains (both constitutive and inducible), or to assess combined gene



disruption to assess gene redundancy. These programmes require large numbers of animals since the probability of generating the correct genotypes decreases with the numbers of alleles involved. Several of our projects involve developing mouse models with multiple genetic modifications.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

My group and I have attended courses on experimental design and statistical approaches and will seek further statistical advice from colleagues whenever necessary. My lab members and I are also aware of online tools such as Experimental Design Assistant (EDA) and G*Power (<http://www.gpower.hhu.de/>).

What other measures apart from good experimental design will you use to minimise numbers?

There is an on-going cryopreservation programme in which valuable lines are frozen. This both secures the lines and allows us to send embryos rather than live mice to other researchers who have the technical capability to receive them.

Breeding programmes are optimised to ensure as little over-breeding as possible. We aim to keep “surplus” animals to a minimum and in the current programme we have averaged fewer than 9% surplus animals.

Where possible we are combining gene reporter alleles to produce compound reporter mice to reduce the maintenance of individual reporter strains.

In order to reduce the numbers of breeding pairs the mice will be kept as homozygous lines (when appropriate and with occasional back-crossing to maintain line integrity), provided that they do not have a harmful phenotype.

To maximise the information from a single animal, we will aim to provide additional tissues to appropriate scientists so that they do not have to breed mice specifically for their experiments.

We have introduced the generation of gene-targeted mice from in-bred ES cells. This removes the necessity to back cross lines for several generations.

Once characterised, we have made our animals available to the scientific community thereby removing the need to generate multiple animal lines with the same genotype.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?



The mouse has been selected for this project based on it currently being the "lowest" mammalian species in which these experiments can be performed robustly and reproducibly. Their immune and haematopoietic systems are the best analogue for those of humans and many models of human disease have been developed in them. The mouse also benefits from well-established and robust technologies for transgenic and gene-targeted genetic alteration.

We are using a number of interventions, which potentially can lead to clinical signs that mimic human diseases including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, viral infection, bacterial infection, cancer, asthma and aging. The vast majority of the procedures that we undertake will be mild with a minority being moderate. In fact we aim to keep suffering to mild in most cases, or failing that moderate since these are more likely to provide us with meaningful information. However, in a very small number of cases e.g. viral and bacterial infections, and experimental autoimmune encephalomyelitis (EAE), it may be unavoidable that the animals develop moderate clinical signs. In particular, this will be the case when we aim to modulate immune responses testing approaches that are intended to reverse rather than prevent the disease. Batches of virus or bacteria, or toxins/drugs may vary in pathogenicity or toxicity, whilst different strains of mice may vary in their resistance or susceptibility. To reduce the risk of unanticipated suffering we will first low dose groups of two mice, a further two mice may be tested at a higher dose and so on until an appropriate dose level is reached. We use a comprehensive monitoring and scoring system to assess the animals throughout experiments.

Where possible we will use the shortest models of disease that will yield satisfactory data.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice are currently the "lowest" mammalian species in which these experiments can be performed robustly and reproducibly. Their immune and haematopoietic systems closely resemble those of humans and many models of human disease have been developed in them. The mouse also benefits from well-established and robust technologies for transgenic and gene-targeted genetic alteration.

We do perform numerous in vitro assays to both define the function of the factor or cells under study, and to refine how we should continue the research so as to optimise the information gained. However, even these assays are better performed with primary cells or tissues ex vivo rather than with cell lines that may have an altered state due to their long-term culture.

The vast majority of the procedures that we undertake will be mild with a minority being moderate. We aim to keep suffering to mild in most cases, or failing that moderate since these are more likely to provide us with meaningful information. Furthermore, we perform airway plethysmography and optogenetics on terminally anaesthetised mice to reduce suffering.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?



We use a comprehensive monitoring and scoring system to assess the animals throughout experiments. This will be refined if/when additional methods of health monitoring are identified as useful and robust.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Surgery will be carried out according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017).

Tumour studies will be undertaken according to the principles outlined by Workman et al., Br J Cancer (2010) 102:1555-1577. Guidelines for the welfare and use of animals in cancer research.

Intrathymic injections will be undertaken with a minimally invasive procedure rather than one requiring osteopathy (de la Cueva et al. 2007. Lab Animal. 36:27).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Our named information officer, veterinary surgeon and facility managers keep us informed of advances in 3Rs that can be implemented.

Explain the choice of species and the related life stages

All our in vivo experiments are performed in mice and many of our other experiments in vitro/ex vivo require cells and tissue from donor mice. Mice are currently the "lowest" mammalian species in which these experiments can be performed robustly and reproducibly. Their immune and haematopoietic systems closely resemble those of humans and many models of human disease have been developed in them. The mouse also benefits from well-established and robust technologies for transgenic and gene-targeted genetic alteration.





63. Immunological mechanisms in pregnancy 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
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Immune cells, Pregnancy, Cancer, Infections

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 is the aim of this project?

To understand how immune cells, in particular those of the female reproductive tract, regulate placental development and how they may be exploited to improve treatment of cancer, with a focus on ovarian cancer.

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

Why is it important to undertake this work?

Our work suggests that interactions between the placenta and maternal immune system are key for normal placental development and fetal growth. The placenta develops from the trophoblast that forms in the blastocyst, an early stage of the embryo. Once formed, it



nourishes the fetus through the umbilical cord. Pregnancy complications like miscarriage and the hypertensive placental disorder pre-eclampsia are due in part to placental dysfunction. The mechanisms of the interactions between the placenta and maternal immune system are unclear, and we seek to determine them. In doing so, we may help understand the nature of pregnancy complications, including those caused by viral infection in the womb. Moreover, we will be able to study immune responses to SARS-Cov-2 during pregnancy in a mouse model of COVID-19 and the potential problems to pregnancy during the infection. Also, we will have the opportunity to test vaccines that may be effective at preventing pregnancy complications linked to the infection.

The immune cells found in tumours and in the placental bed are remarkably similar and similar mechanisms may underlie the growth of cancer and the placenta.

Less than 50% of patients survive 5 years after diagnosis of cancer of the ovary. Due to the asymptomatic nature of the early stages of the disease, most cases are diagnosed in advanced stages. After surgical removal and chemotherapy, the cancer comes back in 70% of cases, so we need to find new treatments.

New immunotherapies are changing treatments of patients with certain types of cancer, like skin cancer. Ovarian cancer spreads primarily to other tissues in the pelvis and the peritoneal lining. A fluid, called ascitic fluid, accumulates in the abdomen when malignant cells spread to the peritoneum. The accumulation of this fluid is called ascites. Ascites contains immune cells, suggesting immunotherapy may be a possible treatment option. Natural Killer (NK) cells are among the white blood cells present in the ascites. NK cells have a natural propensity to destroy tumour cells and our preliminary studies show the presence of NK cells in ascites, suggesting that if we understand the basic mechanisms of how these NK cells work, we may enhance immunity against cancer cells.

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

Better understanding of how immune cells help fetal growth, may protect the fetus from congenital infections and also aid in anti-tumour immunity. Moreover, we will be able to study immune responses to SARS-Cov-2 during pregnancy in a mouse model of COVID-19 and the potential problems to pregnancy during the infection. Also, we will have the opportunity to test vaccines that may be effective at preventing pregnancy complications linked to the infection. Publications of papers in scientific journals. Production of new GA mice for research.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The scientific community will benefit from new understanding of the immune processes leading to pregnancy complications and cancer.

In the long term, data generated may benefit patients and clinicians by contributing to the development of effective immunotherapies, which will ultimately reduce the health and economic burden caused by pregnancy complications and ovarian cancer.

How will you maximise the outputs of your work?



Data will be presented at conferences and published in open access journals. We may also publish according to the ARRIVE guidelines to make sure others either don't have to repeat our experiments or, if they do, they will have all the information necessary to do so.

Species and numbers of animals expected to be used

- Mice: 8500

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The vast majority of the mice will experience only mild and transient discomfort that does not cause any lasting harm, for example blood sampling, mating, and live imaging.

Surgical procedures will be used in a small minority of cases to generate genetically altered animals. For example, some male mice will undergo vasectomy, whereas some female mice will undergo surgical transfer of embryos into the uterus. However, non-surgical procedures may also be used to transfer embryos into the uterus as these procedures are reaching a success rate that matches the surgical procedure.

Some mice may undergo total body irradiation, which is used to prepare host mice for engrafting donor blood cells from another mouse.

Some mice, including pregnant mice, will undergo experimental infection, while others (non-pregnant), will undergo administration of tumour cells.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Surgical insertion of fertilised embryos into the uterus (less than 2.5%) or vasectomy (less than 1%) may cause transient pain, however peri and post-operative analgesia will be provided.

Infection or other signs of ill health (bristling of hairs, subdued behaviour, diarrhoea) may occur in irradiated mice (less than 0.3%) and may only last for a period of 24 hours, after which, if the signs persist, mice will be humanely killed.

Experimental infections and tumour burden may cause signs of ill health. These signs are expected in less than 1% of the mice and may include bristling of hairs and hunched posture, inactivity or lack of appetite, diarrhoea or shortness of breath, and up to 15% of body weight loss - as measured against control, untreated littermates matched for sex and age). These signs may only last for a period of 24 hours, after which, if the signs persist, mice will be humanely killed.

Initial studies in other laboratories suggest that SARS-Cov-2 infections in non-pregnant mice causes only mild symptoms. Because nobody knows how pregnant mice will respond



to SARS-Cov-2 infections, we need something to compare to. For this reason we will also infect mice with agents known to cause pregnancy complications, such as miscarriage or retarded growth of the fetus. These agents include the influenza virus, for example, or a parasite known as toxoplasma. Pregnant mice infected with these agents may experience temporary weight loss and temperature. Mice will be culled before they give birth, so any effect to the unborn progeny will not be carried.

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

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

We expect over 95% of mice to experience only mild and transient discomfort, with less than 5% experiencing moderate suffering.

What will happen to the animals at the end of the study?

- Kept alive
- Used in other projects

Replacement

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

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

We need to use mice because they are essential to evaluate pre-clinically strategies that use immune cells to improve pregnancy outcome and to fight cancer.

The mouse immune system is similar to the human immune system that data we obtain are applicable to clinical research. For example, in both human and mice, a special type of immune cells, called natural killer cells, abound at the maternal-fetal interface, and in both species the type of placentation is invasive. Moreover, new methods to reawaken the immune system in cancer patients have been established in mice and are demonstrating clinical benefits in humans.

What was your strategy for searching for non-animal alternatives?

There are non-animal models of cancers, but we cannot measure how immune cells prevent the spread of cancer in these models.

Some aspects of the tumour microenvironment may be analysed in three-dimensional models of ovarian cancer. Three-dimensional organ cultures that allow the study of some components of the maternal-fetal interface, including endometrium and the tissue that make the placenta, i.e. trophoblast, have also been developed. We collaborate with groups who have developed these systems and, in the future (possibly within the duration of this project), it will be possible to use these models to potentially address the role of immune cells in pregnancy.



We have access to both patient samples, and state-of-the-art monoclonal antibodies. These cells and reagents can be used in test tubes to study some, but not all, fundamental aspects of the interactions between immune cells and cancer cells.

Why were they not suitable?

Uterine immune cells in the body are dependent on their specific environment, which is why animal models are required to understand the fundamental biological processes.

Although there are non-animal models of ovarian and other cancers, we cannot measure how immune cells which kill cancer cells prevent the spread of cancer (metastatic cancer) without using animals.

To translate fundamental biomedical research into clinically useful products or protocols, it is essential to test key hypotheses in pre-clinical 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?

Where relevant, factorial experimental designs are used, rather than the one-thing-at-a-time approach, to maximise the information obtained from the minimum resource. For most of the experiments in which we measure variables, sample sizes are usually set using power analysis, generally using a significance level of 5%, a power of 80%, and a least practicable difference between groups of 25%. Otherwise, previous experience (ours, or from the literature) is used to select sample sizes.

In terms of the numbers of animals required, we expect 6-8 animals per treatment group to be sufficient to obtain the required results. However, because the number of some of the immune cells in the uterus is small, we expect to have to use rather greater numbers of animals per group to obtain satisfactory results. We will use tools such as the Experimental Design Assistant (EDA) which can be found on the NCR3 website (<https://www.nc3rs.org.uk/experimental-design-assistant-eda>).

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We will use tools such as the Experimental Design Assistant (EDA) which can be found on the NCR3 website (<https://www.nc3rs.org.uk/experimental-design-assistant-eda>).

Also, advice on the proposed experimental designs and methods of analysis of the results will be taken from the Statistical Services Unit.

What other measures apart from good experimental design will you use to minimise numbers?



We consider our previous work and experience as well as published work. If we use the procedure for the first time, we run a pilot study. We typically use control animals and blind the experimenter to the genotypes and treatments of the animals.

We will design our experiments guided by the PREPARE guidelines.

We will write a comprehensive Study Plan for each experiment. The Plan will include a statement of the objective(s) and a description of the experiment. This description will cover experimental treatments, size of the experiment (number of groups, number of animals/group), and experimental material. An outline of the method of analysis of the results will also be included in the Plan. This may include a sketch of the analysis of variance, an indication of the tabular form in which the results will be shown, and some account of the tests of significance to be made and the treatment differences that are to be estimated.

We will make appropriate arrangements to randomly assign animals to experimental groups and blind studies and will plan and conduct studies to enable them to be published according to the ARRIVE guidelines.

We will share tissue with other researchers.

We optimise breeding colonies with the help of the Mouse Colony Management System (MCMS) database.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use mice, including embryos, adult and pregnant adult mice.

We will use standard and state-of-the-art methods to produce genetically altered animals, along with specific methods to study immune responses during pregnancy, infection, and cancer. The models and methods we use are those which cause the least pain, suffering, distress or lasting harm to the animals.

For example, herpesviruses (e.g. cytomegalovirus) are the pathogens of choice for the infection models in pregnant mice. While relevant to human pregnancy because cytomegalovirus causes congenital disorders, the mouse cytomegalovirus does not cross the placenta, and only causes mild and transient discomfort to the strain of mice we use. Moreover, to avoid any collateral damage to the offspring, infected pregnant mice are humanely killed before giving birth.

The cancer model we have chosen to use is based on injecting mouse cancer cells into the abdominal cavity of mice. These cancer cells mimic the tumour spread experienced by patients with ovarian cancer, however do not spread further through blood, thus causing



only a mild form of cancer. Nonetheless, with this method we can accurately measure the immune response to the cancer itself, thus furthering our understanding of immunity to cancer.

For some experiments we will administer substances that switch on modified genes (transgenes). Although these substances are generally injected in the abdominal cavity, we can also administer them with the food, which we can flavour to make it more palatable. By administering the substances with the food, we reduce the risk of complications in pregnant mice. This route of administration with the food can also be used when repeated administrations of the substance are required.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We have considered the use of less sentient species, however their reproductive biology is too different from that of mammals to be informative and their immune system is too different from the human immune system to model anti-tumour immunity.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

To improve the quality of life of the animals we keep mice in groups where possible and keep mice singly-housed to a minimum. This reduces contingent harm, stress and stereotypical behaviour. A good use of environmental enrichment is maintained along with minimal stress through restraining. i.e. for intravenous injections. We also do not transport many animals as most of our mice are bred in house and kept in one facility.

We use analgesics to lessen pain.

Scoring sheets are used to monitor the health of animals undergoing procedures.

Mash will be provided for animals likely to lose weight before they start a treatment where weight loss is expected.

We will use infrared thermometers to monitor temperatures to precisely assess potentially raised body temperature as a result of experimental infection. Currently there are no correlative data available but we will collect data with the view of using body temperature as refined criterium to use in this work.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

British Journal of Cancer (2010) May 25;102(11):1555-77
(<https://www.ncbi.nlm.nih.gov/pubmed/20502460>)

LASA guiding principles documents of aseptic technique
(https://www.ubs.admin.cam.ac.uk/files/lasa_aseptic_surg.pdf)

ARRIVE Guidelines for preparing papers for publication (<https://www.nc3rs.org.uk/arrive-guidelines>)



In planning our experiments, we consider the 15 topics of PREPARE guidelines, including formulation of the study, dialogue between scientists and the animal facility and methods (<https://www.ncbi.nlm.nih.gov/pubmed/28771074>).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will follow advances through the NC3Rs website which provides an extensive library of 3Rs resources, guidelines, practical information and themed hubs. Provided are also links to publications, other online resources, and video and training materials.

The internal website also provides a portal to numerous sources of information. Some of these are listed below:

(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 <https://www.nc3rs.org.uk/welfare-assessment>

<http://enrichmentrecord.com/>

<https://science.rspca.org.uk/sciencegroup/researchanimals/implementing3rs/rodentwelfaregroup>

Explain the choice of species and the related life stages

We have chosen the mouse to study immunological mechanisms in pregnancy and cancer, because the mouse immune system is similar to the human immune system. Therefore, data obtained are applicable to clinical research.

We have chosen to use pregnant mice so that we can study the function of immune cells during pregnancy.

Mice are essential to evaluate pre-clinical strategies that use immune cells aiming to improve pregnancy outcome and to fight cancer. The species we have chosen is the one that we believe best mimics human disease.

Prior to embarking on animal experiments, we will collect as much evidence as possible from publications and by using in vitro models of immune cell activation.





64. Immunotherapeutic interventions for cancer

Project duration

5 years 0 months

Project purpose

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

Key words

cancer, immunotherapy, vaccines, cancer-associated fibroblasts

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 is the aim of this project?

The aim of this project is to develop novel vaccines such as DNA vaccines and plant viral vaccines for cancer patients with low tumour-infiltrating lymphocytes with the further focus on combinations of these vaccines and inhibitors of tumour micro-environment such as cancer associated fibroblasts (CAFs).

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

Why is it important to undertake this work?

Cancer is a significant public health issue, with one in 3 people diagnosed, and a quarter of all deaths in the UK attributable to it. In recent years we have witnessed the emergence of immunotherapeutics such as monoclonal antibodies checkpoints inhibitors pembrolizumab and nivolumab which made a significant improvement in the treatment of many cancers. These treatments largely rely on tumour infiltration by T lymphocytes (TILs) and hence, those patients who have low TILs do not benefit from these expensive treatments. In this project we are looking to address novel concepts leading to the



development of novel treatments such as vaccines and therapeutic interventions such as CAF inhibitors which will lead to improved survival of patients with low TILs.

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

data on efficacy and immunogenicity of novel vaccines including next generations of DNA vaccines and plant viral vaccines, and combinational approaches with immunomodulatory antibodies and chemotherapy

identify novel epitopes in target antigens feeding the information to the publicly available databases for epitope identification

generate knowledge on how novel vaccines operate in the setting of immunosuppressive tumour microenvironment

generate novel vaccine candidates for testing in a clinical setting

shed light on immunosuppressive properties of cancer-associated fibroblasts and macrophages in relation to the function of anti-cancer T cells

manuscripts and patents defining the above

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The findings will engage the interest of the following groups: clinical oncologists, scientists interested in immunotherapy, cancer vaccines, vaccines for other diseases including infectious diseases, and biotechnology focused on vaccine development and gene therapy.

On the long term the project will benefit the patients with head and neck cancer and potentially other cancers where the target antigens are expressed such as lung cancer because our novel vaccines target antigens which are expressed in both cancer types. From work on inhibition of tumour microenvironment or combinational approaches we expect that patients with highly fibrotic tumours (CAF high tumours) will benefit. These approaches may be particularly beneficial to patients in oral cancer and triple negative and Her2 positive breast cancers for which there are currently no effective treatments. The project will also benefit patients with myeloma who require long term maintenance therapy. In myeloma, vaccination in remission post-therapy in the setting of minimal residual disease is widely considered the optimal setting for immunotherapy in particular, when disease is de-bulked and immunosuppression markedly reduced. This represents a window of opportunity to develop effective antigen-specific T-cell response aimed at preventing future relapse of the disease. We also expect NHS and health services in other countries to benefit in terms of cost reduction using vaccines and anti-fibrotics and better therapeutic options.

How will you maximise the outputs of your work?

The findings of this study will be disseminated through publications in peer-reviewed journals and presented at national and international meetings.



Results will also be disseminated through the European Cancer Immunotherapy group and the US Cancer Vaccine Consortium.

We participate in well-established public outreach and engagement activities that ensures our research is communicated to the local community including local charities, local branches of national charities and patient groups, as well as the general public. Last year, for example, our local outreach initiatives were attended by more than 50,000 individuals during National Science Week, NHS patient day and cancer research activities. Our group is actively involved in these activities and all PhD students take part in these engagements.

If pre-clinical vaccine testing is successful then an early phase clinical trial will be developed through the NCRI for example. REDACTED. This group forms a central part of the UK's oral cancer research infrastructure, bringing together clinicians, scientists, statisticians and lay members to co-ordinate a strategic portfolio of suitable for publication.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

It is anticipated that a large proportion of mice will receive at least one injection with a therapeutic intervention including novel vaccines or combinations of vaccines and inhibitors of tumour microenvironment, and will be challenged with a tumour. In most of cases mice will receive several treatments following tumour challenge to observe a better therapeutic effect. In some cases the tumour challenge will occur prior treatment. Typically mice will be kept until a statistical significance in the experimental outcome (differences in rate of tumour growth between experimental and control groups) has been achieved. In some cases, where intermediate results such T-cell responses in circulation need to be measured, mice will also be bled weekly.

In experiments to test efficacy of novel vaccines (Objective 1) mice will be vaccinated with a candidate vaccine and will be challenged with the tumour (before or after vaccination), this may be combined with depleting antibody to deplete individual immune cell subsets (such as anti-CD4 or CD8) or cytochrome C (to deplete antigen presenting cells) given on different occasions to reveal the immune mechanisms involved in immune induction or effector functions.

In experiments to test combinational strategies (Objective 2) mice will be vaccinated and will be challenged with tumour (before or after vaccination), CAFs or immune cells maybe be transferred together with the tumour, in some experiments combined with immunomodulatory and/or immune subsets depleting antibodies or cytochrome C given on different occasions to reveal the immune mechanisms involved in immune induction or effector functions or impact of CAFs on individual immune cell subsets. Experiments with CAFs may also include CAFs inhibitors.



Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We expect skin necrosis and ulceration in s.c. models which will be closely monitored. This is limited to the B16 model and is required so the meaningful experimental outcome can be achieved e.g. significant difference in rate of tumour growth between the experimental and control group or tumour free survival. In rare cases in the myeloma model we expect effects on normal behaviour including locomotion and hind-leg paralysis. Mice will be monitored at least daily to monitor signs of adverse effects.

Any signs of slower movement than usual will require more frequent monitoring with a specific attention given to hind legs. Mice slow down and might eventually wobble or drag one leg. The effects on normal behaviour including locomotion and hind-leg paralysis (these are not reversible) will be a humane end point and hence the animal is expected to be terminated within 1h. Inability to close the mouth or chew from the presence of tumour or swelling as a result of the procedure and is not expected to last once discovered. In mammary gland models the tumour is induced on the underside of the mouse hence there is the potential for abrasion injuries. There is a potential for ulcerations in these models. Both abrasion injuries and ulceration will be the end points in mice with mammary tumours. Some mammary tumour models disseminate but metastasis are unlikely with the size of primary tumours we will use. If mice develop metastasis they should be killed. Weight loss may occur for any period providing if it is under 20% of body weight.

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

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

Most of mice will experience mild severity, in some cases (10%) moderate severity will occur.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Immune activation influences multiple cell types across the body concurrently and this cannot be adequately modelled *in vitro* at the current time. Similarly, to study the interactions between an ongoing immune response and a growing tumour, or to evaluate immune-mediated pathology there is unfortunately no viable alternative to *in vivo* modelling except using animals.

What was your strategy for searching for non-animal alternatives?



We are committed to replacing mice where possible and we evaluate immunotherapeutic agents on cell lines *in vitro* when we can. For example, TLR agonists such as plant viral particles will be evaluated in extensive *in vitro* experiments using cell lines transfected with TLRs and antigen presenting cells expressing the respective TLRs isolated from patients PBMCs. For DNA vaccine screening of vaccine prototypes will involve evaluation in HEK293 cells with the subsequent Western blot analysis and only candidates with the highest expression in cell supernatants will be selected for further evaluation of immunogenicity in mice. On the other hand as we understand more how cancer vaccines operate in patients we will consider taking vaccines directly into patients without testing in mice. A good example of this is neoepitope targeting vaccines which have been proven safe and immunogenic in several clinical trials making a good case not to undergo testing in mice but moving directly into patients' setting.

We will also use extensive tissue culture assays to evaluate CAFs inhibitors. We have been developing tumour 3D-organoid and tumour slice models to allow modelling some of the key aspects of CAF-T cell interaction such as T-cell exclusion and mobility for both head and neck and breast cancer. The suitability of these novel model systems is still under evaluation.

Why were they not suitable?

However, the above approaches are not suitable to evaluate whether TLR agonists are capable of inducing T cells responses when delivered with antigen as well as evaluate. Similarly it is not possible to recreate the complexity of the interaction of CAFs with various components of tumour micro environment *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?

Statistical considerations

We have performed Power Analysis to determine group sizes for the types of experiment to be conducted under this PPL using the PS:Power and Sample Size calculation program.

T cell responses - for the study of T-cell responses we estimate that we need to detect a two-fold difference in mean with a standard deviation in each group of 1. A two-sided t-test at the 5% significance level and with 80% power predicts that this requires 5 mice per group. Through experience we find that between 3 and 5 mice per group are sufficient to obtain statistically significant differences; again replicate experiments are performed and data may be pooled if appropriate to strengthen the statistics.

Immunotherapy - we are planning to study changes in tumour burden as the primary endpoint for these experiments. Tumour burden will be monitored periodically using growth measurements and the area under the curve (AUC), post treatment, will be compared statistically using an independent samples T- test between the vehicle control and vaccine/treatment alone groups; and between vaccine alone and vaccine + treatment.



Each experiment will be carried out with equal numbers of control and experimental subjects. In our previous study where a vaccine was found to significantly reduce tumour growth each subject group was normally distributed with a maximal standard deviation of 1233 (mm³/day). This study was used to calculate the sample size required in these experiments. If the true difference in experimental and control means is a 50% decrease, we will need to study 10 tumours per group to reject the null hypothesis (where means of the experimental and control groups are equal) with 90% power and 95% confidence. This calculation was performed using the PS: Power and Sample Size Calculation version 3.1.2. This number will be adjusted to maintain statistical power as these experiments are carried out and more accurate estimates of means and standard deviations are obtained.

Replicate experiments are then performed to confirm reproducibility and when appropriate data may be pooled to increase sample size.

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). These power analyses will be informed by the pilot studies, or our historic studies. Technologies such as 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 the kinetics of tumour inhibition. 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.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We have performed Power Analysis to determine group sizes for the types of experiment to be conducted under this PPL using the PS:Power and Sample Size calculation program

What other measures apart from good experimental design will you use to minimise numbers?

The majority of mice used under this PPL are inbred thereby minimising intra-group variability and allowing reduced mouse numbers for experiments. Small pilot experiments are initially carried out where simple factors such as appropriate dose or route of administration are not clear. Where multiple inter-relating parameters need to be evaluated larger factorial experiments are performed to minimise the number of control mice needed.

The development of new technologies (e.g. multi-parameter flow cytometry and RNAseq) has significantly increased the amount of data that can be obtained from a small number of cells from one individual animal. These technologies are exploited as fully as possible enabling group sizes to be smaller and for more data to be obtained from longitudinal studies (e.g. from sequential blood sampling or tumour imaging) without the need to cull mice to take, for example, the spleen, at each time point.

For subjective measurements (tumour infiltration with immune cells such as T cells), and where possible, scoring is performed by an experienced pathology technician to minimise



bias. For most other measurements (e.g. analysis of cell frequency in tissues by flow cytometry ex vivo and measurement of tumour volume) measurements are objective and blinding is not required. Groups of mice used in experiments are routinely sex and age matched and where possible taken from the same litters. Allocation of mice for experiments is performed blind as researchers request animals which are then supplied randomly from a breeding stock by a technician with no knowledge of the experiment. In addition and where feasible, mice for tumour challenge experiments will be randomised by assigning animals to groups prior to therapy to ensure an equal distribution of tumour mass within each group. This ensures that all animals have detectable tumour prior to treatment and prevents bias. Many of our previous publications conform to NC3Rs ARRIVE Guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>) and we will continue to publish to this standard.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Choice of species/agents

This PPL will use mice as these are the least sentient of the species appropriate for this research. Mice represent a relevant animal model for these studies and the clinical successes now being reported using immunomodulatory drugs or vaccines against cancer were dependent on data arising from such murine studies. In addition, the wide availability of inbred and GA strains and murine specific reagents will facilitate the successful attainment of our research goals and maximise the data obtainable per mouse. Where possible agents to be injected into mice are obtained from published/commercial sources and their dosages and adverse effects (if any) are therefore known. Should substances be used for the first time in mice by us, individual mice will be treated and monitored closely for adverse effects .

Regular monitoring

At least daily monitoring by the PIL holder is expected for all the models. Any animals which are anticipated to be nearing a defined end-point are monitored more closely by the PIL holder. Cages housing tumour-bearing animals near the critical end-stage of disease are marked for special attention. If an animal has reached the humane end-point of disease or is displaying signs of distress/suffering the technicians will either cull the animal or inform the personal licensee that the animal should be culled immediately. Should mice show signs of unexplained distress the NVS will be consulted. Through careful monitoring of established end-points the proportion of deaths is low ensuring that mild (Protocol 1) or moderate severity (Protocol 2-5) is not exceeded.

Humane end-points



Animal death is not considered an acceptable endpoint measure. Our humane endpoints are based on recognised guidelines and chosen to allow evaluate the effect of tested interventions on tumour growth (growth inhibition). Subcutaneous or mammary tumours are easy to monitor by measuring tumour size. Animals with subcutaneous tumours will be killed if tumour exceeds 1.5 cm in diameter (recommended for experiments that investigate therapies) or if the tumour ulcerates with score 4 and above (Table 2, Protocol 2). Animals with mammary tumours will be killed if tumour exceeds 1.2 cm in diameter as larger tumours might impact mobility or tumours might ulcerate. Animals with oral tumours will be killed if tumour exceeds 0.5 cm in diameter, we do not expect ulcerations with our oral models. For oral tumours weight loss of 20% will be a measurable endpoint. For myeloma if palpable spleens are 1.5 cm or an early onset of leg paralysis (problem with mobility) occurs, animals will be killed. Use of *in vivo* imaging should allow refine further our humane endpoints. If tumour interferes with normal behaviour (feeding, drinking, movement), posture or locomotion the animal will be killed in all models.

Anaesthesia

Usually agents and procedures are delivered/performed without anaesthesia to reduce trauma. If required anaesthesia will be administered according to published sources and by persons appropriately trained to do so. If necessary warming mats will be used to aid recovery from general anaesthesia. For longitudinal blood sampling local anaesthetic is applied to the tail prior to bleeding and lubricant used to prevent soreness. Mice will not be subjected to general anaesthesia/sedation on more than 6 occasions. Procedures are combined under the same anaesthesia when possible (electroporation and IVIS imaging for example).

Cell injections

Cells for injection are usually grown under aseptic conditions *in vitro* or obtained directly from mice. Cells will be visualised for signs of infection and washed in PBS prior to injection. Mice receiving new cell lines will be maintained in isolation prior to health screening to prevent transmission of pathogens. The transfer of identifiable cells (e.g CFSE labelled, or congenically marked) is not associated with any adverse effects. Cells for i.v. injection will be visually inspected immediately prior to injection to prevent embolism; efforts will be made to perform injections prior to 3 pm to allow time to monitor mice for signs of embolism and other clinical signs.

Blood sampling

Blood sampling will generally follow Home Office guidelines. Animals may be test bled on up to 5 occasions. Tail bleeding by vein incision with a suitable anaesthetic is the quickest and least stressful method.

Administration of vaccines, antibodies and CAF inhibitors

In general vaccines used in this PPL are well tolerated with no adverse effects. All cytokines to be tested will be injected locally hence no problems are anticipated. Anti-fibrotics CAF inhibitors we intend to use have an excellent safety profile. We established that CAF inhibitors are best delivered by oral gavage. Antibodies (CPI, anti-CD38 and depleting antibodies) we plan to inject are not expected to generate any serious side effects such as a cytokine storm etc. Reagents are prepared under endotoxin low conditions to minimise immune activation which could compromise experiments and induce adverse effects in mice at high dose. Some agents may require dilution in a solvent



other than saline (e.g. DMSO or ethanol). In this case concentrated stocks will be diluted in saline prior to injection to ensure that solvent is low (<10% of volume and <20% volume for detergents as recommended by recognised guidelines. Generally, injection volumes will be kept as low as possible but will never exceed those in recognised guidelines.

Our group has expertise and extensive experience in electroporation to deliver DNA vaccines and in oral gavage for CAF inhibitors.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The scope of this project is to evaluate the novel candidate vaccines/ nanoparticles or tumour micro- environment both required functional immune system of an adult animal. These require interaction of many components of the immune system within the immune system and with the tumour and often require time to mature e.g. the immune response to vaccines goes through several stages including initial activation followed by an effector stage and finally by memory formation. Similarly the tumour microenvironment will take as long as the tumour to develop.

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

low endotoxin preparations of agents for injections, in most of cases the agents for injections will be prepared aseptically

minimise a number of injections if they cause any irritation e.g. oral submucosal/ sublingual injection

use of soft food pellets for 48h after oral injections and when oral tumours become noticeable. The use of one needle/one injection is now a common practice in our establishment

cages housing tumour-bearing animals or other disease models near the critical end-stage of disease are marked for special attention

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Guidelines for the welfare and use of animals in cancer research, 2010.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

All PILs working under this license will subscribe for monthly updates on the NC3Rs website. A dedicated research technician will attend NC3R conferences and will update the PILs under this licence at a lab meeting.

Other on line resources will be accessed including: the European Commission 3Rs databases

https://ec.europa.eu/environment/chemicals/lab_animals/3r/key_resources/databases_en.htm

the European Centre for the Validation of Alternative Methods (ECVAM) search guide:



Explain the choice of species and the related life stages

In this PPL we are interested in developing novel immunotherapeutic strategies to combat cancer we therefore require an adult mouse with a mature immune system to investigate how various component of the immune system interact with cancer.





65. Impact of psychosocial stress on nervous system function and ageing

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Animal types	Life stages
Mice	neonate, adult

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The overall aims of the project are to determine the biological mechanisms by which psychosocial stress manifests physically within the body, resulting in changes to genes, proteins and cells of the nervous system, and the overall behaviour of the organism. This work is undertaken with the long-term objective of understanding the biological basis for stress-induced medical conditions, thereby informing on the developing of disease-modifying therapies for such 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?

We know that the stress response is a conserved series of complex biological processes throughout many species that represents a fundamental mechanism for detecting changes



within our internal and external environments. As such, it is essential for the survival of many species. In most cases, the stress response results in biological changes that allow for adaptive responses to such changes; this is called homeostasis.

However, depending on the degree of stress (acute or chronic), and at what period in your life you experience it (young; old), stress can induce maladaptive responses which confer a risk for developing a range of debilitating medical conditions. Mental illnesses such as anxiety and depression, cardiovascular diseases such as high blood pressure, metabolic disorders such as type two diabetes and immune disorders such as inflammation are all examples of medical conditions that can arise as a result of an impaired stress response.

Therefore, to develop therapies that deal effectively with the causes of such conditions, rather than merely treat their symptoms, it is essential to understand the full biological role of the stress response in altering the underlying processes which lead to the pathology.

We know that the nervous system, the brain in particular, is the key orchestrator of the stress response because it coordinates the activity of different organ systems, thereby allowing for adaptation throughout our body. However, we have yet to fully understand the pathways which result in processes altering the genes and proteins that determine the activity of cells composing the various stress networks throughout the brain and various organ systems. We also do not know why certain forms of stress are beneficial and lead to adaption, whilst other forms confer a vulnerability to disease. Knowing this could help to develop strategies that provide protective measures in vulnerable individuals, thereby preventing the onset of such conditions in the first place.

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

The primary outputs will be:

New scientific information that contributes to our greater understanding of stress biology and how this contributes to various diseases, will be disseminated by publishing in peer-reviewed scientific journals and by presentations at scientific conferences and Lay public events.

Scientific justification for future drug development studies focused on treating stress-induced medical conditions.

Patents for new drug targets.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

SHORT TERM:

Basic biomedical scientists and clinicians in various medical disciplines (neurology; gastroenterology; metabolic diseases): advancement of basic neurobiology knowledge and how this underpins the pathology and symptoms of medical conditions.

Drug development scientists: identification of novel drug targets for potential translational exploitation.

LONG TERM:



Healthcare policy makers (e.g. NICE): translation of the basic scientific knowledge into and the new pharmacological therapies it give rise to, will be used in the rational decision-making for optimal healthcare policies.

Patients suffering from various stress-induced disorders will benefit from personalised drug therapies which are capable of addressing the fundamental causes of their condition, thereby modifying the core disease, rather than simply treating the symptoms.

How will you maximise the outputs of your work?

All this work is conducted with external collaborators as well as Lay research monitors associated with my Funders. Therefore, I will use these extensive networks to disseminate the knowledge. I will strive to publish the data, both positive and negative, in the most widely read scientific journals.

Species and numbers of animals expected to be used

- Mice: 8000
- Rats: 1000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Broadly, animals will be exposed to stress, primarily by altering their interactions with other mice.

The project can broadly be divided into two phases, namely the induction of stress in mice and then the investigation of the biological consequences of the stress responses in these mice

INDUCTION OF STRESS:

For early life stress, the behaviour of the mother will be altered which results in a fragmented mother- pup relationship. This impairs the quality of the care she imparts to her offspring, resulting in an enhanced stress response in adulthood.

For adult stress, test mice will be exposed to a more aggressive strain of mouse which results in social subordination and a stress response.

ASSESSMENT OF STRESS RESPONSE

To determine what consequences stress has on a range of organ systems, a range of techniques will be used, for example:

Changes in physiology: under anaesthesia, brain activity and how it changes in animals exposed to stress will be determined using standard recording techniques.



Changes in genes, proteins and cells: animals will be killed humanely and the stress-induced changes in a variety of biological processes, within different organs, will be determined.

Behavioural assays: stress induced changes in native mouse behaviour will be determined using a range of behavioural tests to assess any anxiogenic/depressive-like behaviours.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

All the adverse effects will be as a result of their exposure to stress. This can result in changes in:

Behaviour: animals may exhibit an increased fear response, for example agitation to normal handling.

Metabolism: animals may have altered body weights due the effects of stress on various hormones or their motivation to feed.

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

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

The majority of the animals will be used in Protocols 1 and 2, and subjected to some form of stress. Therefore, for the majority of animals, the most extreme level of severity will be categorised as Moderate

However, a significant proportion of the experiments fall into the non-recovery category because they will be conducted under terminal anaesthesia with the animal being unconscious throughout and therefore insentient during the actual experiments.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

The objectives of the project are to elucidate which life experience, in the form of stress, alter the coordinated functioning of various body systems, such as the brain and peripheral organs, resulting in changes in the behaviour of the animal.

The research questions necessitate the use of native animal tissue since the anatomy, physiology and neurochemistry of different neuronal circuits in specific parts of the brain and native animal behaviour can only be elucidated by studying the intact animal itself.



Furthermore, to investigate the changes that occur in brain function due to conditions such as stress or depression, it is necessary to expose animals to such conditions and then determine the consequent effects on the brain itself.

What was your strategy for searching for non-animal alternatives?

Cell-culture: It is possible to assess the impact of certain brain proteins on cellular activity by expressing the proteins in cultured cells.

Computation neuroscience: as scientific knowledge advances, there is a growing availability of computational models of brain function.

Why were they not suitable?

Brain function, and the associated diseases, arises from a multitude of individual cell types (millions).

It is simply not feasible to faithfully replicate the molecular, morphological and functional complexity of these different brain cells, in basic in vitro cell system.

Modelling: in order to get robust models, it is essential to feed robust primary data into the various algorithms. The lack of available primary data regarding the effect of stress on the parameters under investigation in this project precludes the use of such technology at this stage.

Reduction

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

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

The estimates are largely based on the numbers used during similar experiments for my last two Project licences. However, the following refinements have been added:

Power calculations: when pilot data provided an effect size, I used this to calculate the optimal sample size for individual experiments.

In all case, sample sizes for individual experiments have been calculated using the following parameters:

Power: 90%

Alpha: 0.05

Population Variance: according to the parameter being assessed

However, some of these experiments are novel and at this stage, it is unclear what the outcomes will be, therefore precluding the identification of an effect size, and thus power



calculations. In such incidences, we have been guided by the minimal number animals normally required for such techniques, e.g., histological, $n = 5$; gene arrays, $n = 8$.

Finally, we have taken into account the additional animals required when experiments fail as a normal consequence of scientific research.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

I have made use of all local support including our statistician and other resources such as the ARRIVE or PREPARE guidelines.

What other measures apart from good experimental design will you use to minimise numbers?

Together with my collaborators, we will use our experience, demonstrated in numerous publications, to use the minimum number of animals which are required to provide scientifically valid data. Since my collaborators also work on body systems other than the brain, we will utilise multiple organ systems from individual animals thus resulting in a reduction of the total number of animals used.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

I will be using mice and models of psychosocial stress in:

Adulthood: social defeat model of chronic stress

There are a range of models of chronic stress, which invariably require the animal to be exposed to extended periods (~ 6 hours per day for 20 days) of aversive experiences. The model I will employ exposes the animal to a conflict situation with another mouse which can last anything from 30 seconds to 2 minutes, after which the animal is removed from the resident compartments. This is repeated for up to 10 days. As such, the model is highly refined to allow for the induction of psychological stress with the minimum amount exposure to stress.

Early life stress (ELS): limited nesting and bedding mode.

Again, a range of ELS models require the exposure of the mouse pups to extended periods of separation from their mother, during the very vulnerable first two weeks of life. The model I will employ does not alter the amount of time the mother spends with the pups. Instead, the model replicates the importance of the quality of maternal care, rather than the amount of maternal care. As such, the model directly addresses the most important factor in terms of ELS, without induces any additional harm to the animals.



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The aim is to understand how the perception of stress, at the mental level, alters the functioning of various body organs. As such, for most of the project, it is important that a certain level of sentience is required to mentally perceive the adverse experience, so that the emotional limb of the stress response, rather than a basic reflex reaction is elicited. This therefore precludes the use of other animals such as fish or amphibians.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

At all stages of the project, experiences will be monitored to determine whether aversive procedures can be minimised without adversely affecting scientific outcomes.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

I will seek out the guidance of my NACWO and fellow scientists in the field in order to ensure measures of refinement are always optimal.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I will maintain an up to date knowledge of advances in the field, using the conventional routes of scientific articles and conferences, alongside local expertise in the form our NACWO and Named Veterinary Surgeon.

Explain the choice of species and the related life stages

The overall aims of the project are to study how emotional stress alters the functioning of various mammalian organ systems.

Mice will be used as the broad anatomy and physiology of this species is well understood and provides the best compromise in terms of correlating with human biology whilst exhibiting the lowest level of sentience.

Since the aim is to understand the effects of stress at different periods of life, it is imperative to use animals at different ages. Therefore, we will use both neonatal and adult mice.





66. Improving the Clinical Outcome of Cutaneous Wounds

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life Stages
Pigs	adult
Rat	adult
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

This project aims to address the clinical problem caused by wounds that are difficult to heal (due to chronic disease such as diabetes, cancer, or physical trauma) by developing a range of materials and therapies that will target specific elements of the wound healing cascade and will speed up the wound closure and healing.

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

Why is it important to undertake this work?



Non-healing wounds are a significant and growing clinical problem. The cost of non-healing/chronic wound care is estimated to consume at least 6% of NHS expenditure in the UK, and many are still left with chronic wounds that simply won't heal using current treatment options causing distress and very poor quality of life.

Another major issue is burns. In the UK alone there are 13,000 hospital admissions every year due to burns, of which 1,000 are due to severe burns. The initial insult is often followed by on-going traumatic experiences with continued treatment, including reconstructive operations, disfiguring scars and compromised functioning of affected parts of the body.

The costs to the patient are much harder to quantify but should be considered in terms of physical and emotional pain and associated psychological damage.

Therefore we will investigate a range of novel technologies/ products for wound healing that can: 1. Increase the rate of wound closure; 2. Reduce scarring; 3. Reduce infection; 4. Improve the quality of the wound healing as a whole.

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

Our dermal replacements have been designed in different formulations to attend different patients' needs. We are developing products that is very easy to apply into the wound void to assure contact with the entire topography, maximising the chances of successful healing. We prioritise the use of high quality products, yet using inexpensive materials, which may reflect in an affordable and off-the-shelf products with different clinical applications.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The products we are developing intend to be high quality products, yet affordable and off-the-shelf, with potential to treat many debilitating conditions, particularly those wounds which simply won't heal, aiming to help elderly people and those with chronic conditions such as diabetes, cancer and burns. We aim to reach the market in the next 3-5 years.

How will you maximise the outputs of your work?

A supportive network of clinicians is accessible to our research hubs, and they are actively involved in many current research programmes. This network as well as patients' support groups have been invaluable in focussing previous projects to enable clear patient benefit.

We have collaborations with various Hospitals in London which provides invaluable advantages for this translational research. We are also academically linked with the Universities, which places our research at the interface between strong academic basic and clinical sciences.

The Institute has a strong track record in scientific publications and training young scientists and doctors in the field of Tissue Engineering/ Regenerative Medicine through the research we develop here.

This work will be disseminated among the academic public by conferences, abstracts and full research articles publication, as well as amongst the public and patients.



This work will be discussed with our collaborators and local support groups for patients and families suffering with non-healing/ chronic and burn wounds in order to be as assertive as possible and have a real patient-focus approach.

Species and numbers of animals expected to be used

- Pigs: 150
- Sheep: 0
- Rats: 150
- Mice: 150

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

We intend to create small wounds on each animal, allowing a large amount of data to be produced while using a minimum number of animals. The wounds will be treated with our skin replacement materials and followed up rigorously for 8-12 weeks. The wounds will be monitored weekly with assessment for infection, inflammation and dressing changes.

Suffering to the animal will be minimised through the use of anaesthetics and well-established analgesia and protective dressings which reduce any pain associated with the creation of the wounds.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

From our experience with these models we expect an uneventful recovery from surgical procedures under general anaesthesia and that post-operative pain will be controlled by use of analgesics. Aseptic technique will be used for all surgical procedures to minimize unintentional infection.

Use of electrocautery at surgery will minimise risks of postoperative haemorrhage. Insertion of wound chambers is usually painless and we have not seen any problems in animals with chambers during the previous studies. Animals will be administered with regular postoperative analgesia (e.g. temgesic) for 48h, and additional analgesia if they appear to be in pain (e.g. diminished appetite, immobility, changes in defaecation or urination, abnormal vocalisation, general loss of condition).

In all animals any abnormal signs (e.g. diminished appetite, immobility, changes in defaecation or urination, abnormal vocalisation, excessive wound infection, general loss of condition) will be investigated by the personal licence holder who shall remain responsible for the animal whilst increasing the frequency of monitoring. The vet will be consulted and their advice taken as to minor remedies or major interventions. This may restore the animal's wellbeing in a short timeframe. Minor remedies may include cleaning and re-



dressing an exposed wound or the administration of antibiotics if this is experimentally appropriate.

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

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

Procedures will be mild (protocol 1) and moderate (protocol 2) in severity and analogous to minor routine surgical procedures performed clinically on human patients. Such procedures include removal of cancers and treatment of moderately sized burns with skin replacement biomaterials and are now routinely undertaken as day cases with no overnight hospital admission.

In the unlikely event of any abnormal signs that are likely to exceed moderate severity – prolonged vocalisation, sustained excessive temperature increase, failure to eat or drink, vomiting, excessively suppurating wounds or if a systemic infection occurs –or if the animal fails to respond to treatments in the expected time the animal will be killed by a Schedule 1 procedure.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

In vitro systems utilising the cell populations responsible for wound repair have been considered and are used extensively as alternatives to the use of animals. Additionally, discarded tissue has been used by our group as an ex vivo model which replicates the architecture of intact skin.

In order, however, to further develop therapies for clinical use an in vivo model must be used to replicate the complexity of wound healing in a clinical setting. This is particularly important where vascularity, immune function, tissue durability and host interaction are required to be examined experimentally.

What was your strategy for searching for non-animal alternatives?

All initial toxicity and screening work was undertaken using well established in vitro or ex vivo models prior to the final validation.

Why were they not suitable?

Whilst both in vitro and ex vivo methodologies provide substantial information regarding the response of specific cells to materials or therapies, the response of a whole organism to wound healing therapies cannot be accurately replicated using existing techniques.



We can assure that only a small number of potential treatments for each study element will be taken forward for in vivo testing thereby ensuring that the number of animals used is as small as possible.

Reduction

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

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

We use the online tool "Experimental Design Assistant" / NC3Rs

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Prior to any tests, during the experimental design phase, we use online tools available (Experimental Design Assistant). An extensive library of online resources is available on the NC3Rs website to provide researchers and technical staff with the practical tools needed to put refinement into practice (<http://www.nc3rs.org.uk/3Rs-resources>).

What other measures apart from good experimental design will you use to minimise numbers?

All the preliminary tests for toxicity and biocompatibility will be performed using in vitro methods.

We intend to create multiple small wounds on each animal, allowing a large amount of data to be produced while using a minimum number of animals.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Collection of small biopsies and/or abdominal fat for cell extraction for future implantation

Creation and treatment of cutaneous wounds/burns.

Suffering to the animal will be minimised through the use of anaesthetics and well-established analgesia and protective dressings which reduce any pain associated with the creation of the wounds.



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Less sentient animal species do not provide the necessary data for treatments to be progressed to human clinical use. The healing process that will be tested require full organism physiology.

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Our local NACWO will be consulted prior to any procedure. At any sign of pain, suffering from animals we increase monitoring and our vet will be consulted and their advice taken as to minor remedies or major interventions. This may restore the animal's wellbeing in a short timeframe. Minor remedies may include cleaning and re-dressing an exposed wound or the administration of antibiotics if this is experimentally appropriate.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

An extensive library of online resources is available on the NC3Rs website to provide researchers and technical staff with the practical tools needed to put refinement into practice (<http://www.nc3rs.org.uk/3Rs-resources>).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Through constant literature review and update. We also take part on webinars and technical updates from NC3R regularly.

Explain the choice of species and the related life stages

It has been shown that pig skin demonstrates many of the physiological, anatomical and biochemical properties of human skin so that any information obtained in these studies is of direct relevance to the way patients are treated.

Lower/smaller animal species do not provide the necessary data for treatments to be progressed to human clinical use though rats may be useful for some studies such as the testing of infection- signalling dressings, for example.

Previous work using similar approaches have been done for many years in our Institute and we never experienced failure or animal suffering.





67. In vivo taste assessment of pharmaceutical compounds and formulations

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

The aim of this project is to provide and use the rat brief-access taste aversion (BATA) test as a scientific service to assess the taste of pharmaceuticals (e.g. for young children) at the early phase of development.

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

Why is it important to undertake this work?

By nature, a medicine cannot be expected to taste good. The active ingredients, which include acids and bases that allow medications to do their job, are often bitter or even unbearably salty bringing an offensive taste, which can be exaggerated by the dose.



Bitter taste is one of the basic taste modalities. Humans' distaste for bitter flavour may have evolved to protect against accidental poisoning. In fact, many potentially toxic substances taste bitter. Bitter is innately aversive for various species and each animal lives in its own "taste world".

Human subjects are the gold standard of taste analysis especially with nontoxic compounds. However, it is not always possible to use human subjects for taste analysis - for example, when assessing new chemicals without complete safety information, which is often the situation during the early stages of drug development. Although research is continuing in the area, it is complicated to use computer models or laboratory-grown cells, rather than live animals, to reliably look for "bitter" responses, as numerous different compounds, with different solubilities and at different doses, can all elicit "bitter" responses.

Using the rat brief-access taste aversion (BATA) test, taste of active pharmaceutical ingredients (APIs) which are intended for oral, intranasal and inhalational administration, can be evaluated during the early stages of development, ideally at the pre-candidate stage. Therefore, it is possible to screen and select the best compound molecule which can be more effectively taste-masked in future development stages. The rat BATA test can provide data which can assist companies to justify the appropriateness of formulations of drugs, for example medicines for children.

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

Data will support product development, product registration, patents, and ultimately lead to new products.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

For research Sponsor companies, data can assist elimination of taste-aversive candidates prior to further costly development, and further animal research.

The model can accelerate development: the BATA is a decision-making support tool.

In the long-term the BATA will benefit patients with various diseases or conditions: the impact of this work is applicable in multiple treatment fields.

How will you maximise the outputs of your work?

Whilst publication of results would not be discouraged, it is expected that in most cases publication or dissemination will not be possible as the research will be conducted as a commercial service to companies.

Species and numbers of animals expected to be used

- Rats: 200

Predicted harms

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



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The rats will be placed in a box on a "Davis rig" ("lickometer"). The lickometer presents different bottles containing different solutions to the rat, and the rat is able to drink from each bottle for 2-8s, then another bottle, with another solution, is presented. This present-drink-present-drink cycle repeats until the rat has drunk from each bottle at least 3-4 times. The process takes up to 2 hours. The rats are placed in the machine once a day for up to 7 days (usually 5 days), which counts as an experimental period. The first 1-2 days are to get the rat used to the lickometer, and then the next 1-5 days are used for experiments with test substances. The first bottle to be presented is always water, and there is a water "rinse" bottle presented between each test substance. The machine detects how many licks per second the animal takes from each bottle. The aim is to detect "taste aversive" substances: substances that the rat really dislikes. To get a reliable "lick per second" rate, and to truly detect aversiveness, we need to ensure that the rat is thirsty, so the rat will not be allowed to drink for 22 hours before each lickometer session. After an experimental period (up to 7 days) has been completed, the rat is allowed to recover with free access to water for at least 7 days, then it can be used again for another experimental period. We plan to use groups of 10 rats for multiple experimental periods on an approximate week-on/week-off basis for up to 6 months.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The rats will be deprived of water during the experiments, so we expect to see signs of dehydration, like some weight loss or skin "tenting". We expect these signs to be short-lived, and only visible during the experimental days. We would expect the rats to recover from these (for example gain weight, or normal 6 month growth) during the recovery period between 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 species)?

We expect all the animals to show mild signs of dehydration during the experiments. Rats have been shown to be very tolerant to these extents of water deprivation, and this particular research group has never had to kill a rat due to dehydration whilst developing the model.

What will happen to the animals at the end of the study?

- Used in other projects

Replacement

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

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



The aim of the BATA is as a screening tool for assessment of taste-aversiveness at the very early phases of drug development. At this stage of formulation development, a lack of sufficient toxicology data prevents the use of humans to assess the taste of new molecules or formulations.

What was your strategy for searching for non-animal alternatives?

"e-tongue"

Why were they not suitable?

The e-tongue *in vitro* tool uses biological sensors and has not been fully correlated to human taste yet.

Reduction

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

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

There will be 10 rats per group, and 2 groups of 10 rats will be used for 6 months at a time. The licence period is for 5 years.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

By repeatedly presenting test substances alongside water over multiple days; each rat is an individual control, eliminating the need for a control group.

The group size was optimised during the development of the model. Re-using animals for multiple experiments reduces total animal use.

What other measures apart from good experimental design will you use to minimise numbers?

Re-using animals for multiple experiments reduces total 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?



Rodents are most often used as subjects in taste research. In particular, rats and mice have been widely used for taste perception studies for many reasons: their high drinking capability, their taste similarity to humans and the large corresponding literature available on the comparison of humans and rodents, and their short lifespan. Therefore, all our protocols and equipments will be optimised for these species.

Several animal models exist for the taste assessment: the more commonly used are the two-bottle taste preference and the brief-access taste assay (BATA). With the two-bottle taste preference test there is a higher risk of the animal receiving a larger dose of the test substance, as the animal has access to the solution for longer. This could then cause side effects and may affect the drinking rate. The two-bottle method also takes a long time to acquire enough data for a dose-response assessment. The BATA test is a better model for our aims, as it lowers the risk of side effects and gives a clearer indication of taste-aversiveness, as well as having robust scientific background and supporting evidence.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

A smaller, less sentient rodent species, such as the mouse, would be less tolerant to water restriction. A younger less mature rat would be less tolerant to water restriction and less capable of accessing the water provided during the tests; the growth rate of immature life stages would also introduce an extra variable to experiments reducing the validity of results and most likely result in using more animals.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The rats will be trained so they can get used to the lickometer. The training takes place in stages: first the rats are placed in the box with one bottle of water, and no moving bottles. The next day, the bottles will move and be presented separately, and all will contain water. The first "experimental period" for new groups of rats may include the use of a substance which has already been assessed by this study, such as quinine or caffeine. As well as being useful for training the rats, including known substances in this initial experimental period can also provide baseline information: this experiment can be repeated during the 6 months that the rats will be recruited to the study, and the results can be compared to their first try. This helps us check that the rats are behaving in a similar way at different times (for example from month to month).

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Our facility is GLP-certified and experiments can be performed to GLP-certified standards where required; non-GLP studies are performed to the same standard operating procedures.

The PREPARE guidelines cover many topics that have been discussed whilst developing this collaboration between the research team which developed the model and our facility, and an adaptive approach to these will be useful for planning specific studies and continuing the project.



How will you ensure you continue to use the most refined methods during the lifetime of this project?

Staff actively seek opportunities for training and continuing professional development. We circulate the NC3Rs newsletters and take part in IAT and LASA activities. We communicate openly about animal welfare and suggestions and ideas for improvements are welcomed from all staff members.

An associated research team is in projects investigating taste aversiveness, including development and validation of alternative models, under a separate project licence. These developments can be incorporated into this service as they are validated.

Explain the choice of species and the related life stages

Rodents are most often used as subjects in taste research. In particular, rats and mice have been widely used for taste perception studies for many reasons: their high drinking capability, their taste similarity to humans and the large corresponding literature available on the comparison of humans and rodents, and their short lifespan. This test has been developed and validated using adult male rats.





68. Increasing the success of glaucoma filtration surgery

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Animal types	Life stages
Mice	adult
Rats	adult
Rabbits	adult

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The main objective of this project is to develop new treatments to minimise scarring in glaucoma. This will include new wound healing modulating drugs, new devices in glaucoma surgery and improved drug delivery techniques in the eye.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 large unmet clinical need being addressed in this project, with glaucoma being the leading cause of irreversible blindness and affecting over 70 million people worldwide. Glaucoma filtration surgery is the mainstay of surgical treatment in glaucoma, but it fails



primarily due to scarring in 50% of patients after 5 years of follow-up. The toxic anti-cancer drug, mitomycin-C, is the current gold standard to prevent scarring in glaucoma filtration surgery, but it leads to severe adverse side effects such as tissue damage and severe infection.

This project aims to develop new treatments to prevent scarring in glaucoma patients. In addition, new improved animal models will increase our understanding of the disease mechanisms of complex wound healing in glaucoma surgery. This project will also help us to identify new therapeutic targets and to develop new drug delivery techniques and devices that could benefit millions of glaucoma patients in both developed and developing countries.

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

Improved drugs to prevent scarring after glaucoma filtration surgery in adults and children. This will lead to more successful surgical outcomes in glaucoma.

New glaucoma drainage devices that allow for better flow control and therefore prevent potentially blinding post-operative complications.

Refined slow release drug delivery methods to improve wound healing post-operatively and the surgical outcomes in glaucoma.

Increased understanding of the disease mechanisms of complex wound healing in the eye and identification of new therapeutic targets and biomarkers.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Patients (next 5-10 years)

Our main impact goal is to increase the success rate of glaucoma surgery and to decrease preventable blindness from glaucoma. New anti-scarring drugs, drainage devices and improved drug delivery methods will lead to reduced complication rates and hospital visits, thereby significantly improving the quality of life of glaucoma patients and their families.

Health Professionals (next 5-10 years)

A new anti-scarring treatment will help ophthalmologists to better treat their patients, by increasing the surgical success of glaucoma surgery and without exposing them to the risk of toxic anti-cancer drugs like mitomycin-C.

3. Socioeconomic and Decision-makers (next 5-10 years)

A reduction in the side effects of the current toxic anti-cancer drugs will lead to less post-surgical complications, including reduced hospital visits and rate of re-operations, thereby saving costs for healthcare providers (NHS cost savings through post-surgical complications of over £40,000 per patient). On average, a patient with complications is hospitalised 1.2 - 3.8 days longer than those with no complications, costing an average of £1,711 - 4,225 including drugs, clinical costs and rooms.

4. Academic and Scientists (short-term, next few years)



The results and databases will be published in open-access journals so that the research will also benefit the scientific community.

How will you maximise the outputs of your work?

I will publish our methods and results in open-access journals so that the research will benefit the scientific community. Fibrosis is a multisystemic disease and I am part of the REDACTED (Inflammation, Tissue Repair, Scarring & Fibrotic Diseases) consortium. I will present our results at the annual REDACTED meetings and at national and international conferences. I will also set up collaborations with other researchers working on fibrosis in the eye and other parts of the body.

Species and numbers of animals expected to be used

- Rabbits: 1000
- Mice: 1000
- Rats: 1000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

REDACTED

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

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

Moderate severity for all animals (100%) undergoing experimental glaucoma surgery.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Although we make extensive use of in vitro (laboratory-based, cell culture) models in this project, there is currently no effective way to model pathological scarring in glaucoma surgery outside the living animal. The new treatments must be able to prevent scarring in



vivo (in living animals) to be successful in the clinic and the use of animals in this project is therefore essential to achieve this aim.

What was your strategy for searching for non-animal alternatives?

In vitro (laboratory-based, cell culture) models, e.g. collagen contraction assays, cell adhesion studies, cell viability assays.

Why were they not suitable?

Due to the complex nature of the wound healing process, in vitro studies alone only provide small clues to what may happen in the entire organism. To this end, only in vivo (in living animals) models can provide the accurate and complete results we require before taking our work forward towards human clinical trials.

Reduction

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

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

During this project, we will use the minimum number of animals to achieve our scientific aims. This will be done by conducting statistical power analysis prior to initiating experiments to ensure that we only use the minimum number of animals required to produce valid statistical comparisons between the experimental groups. Based on previous studies, we will use 6 animals per experimental group as this is the minimum number of animals required to reach statistical significance.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We will use the NC3Rs' Experimental Design Assistant to ensure that we use the minimum number of animals to meet the scientific objectives of the project. We also collaborate with statisticians and they will help with the sample size calculation and experimental design.

What other measures apart from good experimental design will you use to minimise numbers?

Our decision to use wound modulating agents or devices will be guided by extensive in vitro (laboratory-based, cell culture) studies. We will also conduct small pilot animal studies to give us an indication as to the efficacy of the treatment, therefore aiding in the design of the experiments to use the minimum number of animals.

Refinement

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



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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The animal model used is of the least severity that is able to deliver the science and in this case is moderate severity in nature due to the surgery involved. We will minimise animal suffering with the use of anaesthesia during each surgical intervention. We will always give analgesia after surgery. We will also limit the number of times an animal undergoes a drug dosing and animals will be kept for the minimum duration required.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The animal models have been designed to closely reflect human disease. The rabbit model is an established model of eye scarring and agents that have reduced scarring in the rabbit have been shown to be effective in humans in clinical trials. The mouse and rat models are also validated models of eye scarring.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

To minimise suffering, we will administer appropriate anaesthesia for every surgical intervention. Analgesia will always be given to all animals before surgery, during surgery and for as long as required after surgery. We will also monitor the animals carefully in the post-operative period and any animal deemed to be suffering will be removed from the study by a Schedule 1 method.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow the NC3Rs guidelines on the 3Rs (Replacement, Refinement, Reduction) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, and the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I am subscribed to the NC3Rs newsletter and get monthly updates about new advances in the 3Rs. I will contact the local NC3Rs Regional Programme Manager for advice during the project.

Explain the choice of species and the related life stages

Where possible, mice or rats (adults) will be used in this project but due to their small size, certain techniques of glaucoma surgery and examination are extremely difficult, and in such cases, rabbits (adults) will be used instead. The rabbit model is a validated model of eye scarring that has previously helped in the translation of the anti-cancer drug, mitomycin-C, in glaucoma surgery.





69. Individual variation in the life histories of fish

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

No answer provided

Animal types	Life stages
Salmon	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 is the aim of this project?

The project will assess the survival, growth, migration and metabolic patterns of wild, free-living fish; thus, they need to be caught in the wild. As transporting them to a Licenced Establishment is judged to be potentially harmful to their welfare, all procedures will be performed on the fish close to their site of capture (under POLE regulations) so that they can be returned to their capture site quickly after they have recovered from the procedures.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 biology and behaviour of many fish species are increasingly disrupted by environmental changes of both natural and anthropogenic origin. Fish have high ecological significance in rivers (often being apex predators), high economic value (in terms of income from commercial fisheries or angling) and a high societal and cultural value (public appreciating that fish, especially Atlantic salmon, are present in local rivers). Understanding how fish biology and behaviours are affected by environmental changes,



how these might cause fish population changes, and how we might mitigate for any negative impacts, is paramount in the face of ongoing environmental change. Developing this understanding is particularly urgent for Atlantic salmon, brown/sea trout and grayling whose numbers are at their lowest for decades. For example, 94% of Atlantic salmon populations in England and Wales are now classified as at risk or probably at risk. In 2018, the numbers of adult salmon returning to Scottish rivers were the lowest ever recorded.

National and International organisations have expressed major concerns and stated that “Factors other than marine fisheries, acting in freshwater and in the ocean are contributing to continued low abundance of wild Atlantic salmon.” These freshwater factors can include in-river habitat change (e.g. removal of water or in-river plant cover), barriers (e.g. weirs and hydro-electric dams), etc., and will likely affect juvenile and adult growth and survival rates in the freshwater, and even at sea. In 2015, a national regulatory body developed a five-point approach to recover salmon populations in which they prioritise investigating the freshwater factors affecting juvenile and adult salmon life-stages.

Populations of UK wild salmon, sea trout are at their lowest ever levels and under pressure from a range of natural and anthropogenic issues. Our work aims to gain better knowledge and understanding of these pressures, which can be used to recommend and formulate policies to strengthen the abundance and resilience of these threatened populations. To this end, we have involved policy makers in the planning and development of our projects.

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

The expected outputs from this work will include :-

Novel information on life history strategies of individual fish, and consequences for their populations. The individual based knowledge that we will obtain from the PIT tag work proposed under this project licence application is exactly the kind of information that we require to enable us to understand and predict the consequences of current and future changes in the environment at the population level. To obtain enough data to analyse the marine phase of the salmon lifecycle as proposed in this project licence application we need to PIT tag enough juveniles to account for the fact that only 7-12% of the juveniles tagged in the autumn are observed emigrating from the river and only 3-6% of those return from the marine migration.

An improved understanding of the habitat and existing and emerging effects of human activities on populations of these fish. The data obtained from this project licence application will provide novel information on the effect of habitat and anthropogenic pressures on growth and survival. From this we will produce recommendations for management and policy changes for freshwater where appropriate to protect and strengthen declining salmonid populations

Novel information on the movements and survival of migratory fish through estuaries and the near shore. There is a real void of information about the behaviour of migratory salmonids in transitional and coastal waters and the regulating authorities are looking for more information to enable them to implement knowledge-based management of salmonids in this environment. The regulating authorities are actively engaging in the work proposed under this project licence application and the outputs will influence future policies providing better protection of these species currently in decline.

Novel information and improved understanding will be presented in scientific publications and data will be provided for international evaluation of salmon stocks, where appropriate.



Scientific publications will ensure that benefits from improved understanding of salmonid stocks is extrapolated beyond the local setting of this project. Data obtained under this project licence application will also feed into the evaluation/recommendation on high sea catches of Atlantic salmon.

Communication with stakeholder and the general public in the form of presentations and publications, where appropriate. Impacts on policy will help these populations in decline but only by communicating the results obtained under this project licence application and the resultant recommendations for management to stakeholders and the general public can we maximise the positive effects for salmonids and the environment they inhabit.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Short term benefits – years 1-3

National and international governments will benefit from the population abundance data we collect on salmon and sea trout. This information will forge national and international policy for the improved management of these fish.

National bodies will benefit from data we collect on fish tagged during our current project licence. This information will direct policies on river management and coastal planning and netting.

As a research and education institution, we will benefit from continued use of current infrastructure, staff skills and experience, and use them to educate public and stakeholders nationally and internationally.

International collaboration through the working groups on salmon and trout

Long term benefits – years 3-5+

Contribution to historical baseline data used for national and international salmonid stock assessments and through which to measure the effectiveness of policy changes.

New and improved policies for the better protection of salmon and sea trout from coastal netting and coastal planning.

New and improved models to better manage salmon and sea trout stocks for their protection and conservation

Novel information for policy makers on the emerging effects of human activities on salmon, sea trout and grayling.

Improved abundance of salmon and sea trout through the implementation of the above

How will you maximise the outputs of your work?

We have a track record of ensuring our work has a practical application.

We collaborate very closely with influential government organisations and conservation NGOs and they have helped design the hypothesis of our work here to ensure it is relevant and accessible to them to better protect these fish.



We hold regular meetings and sit on groups for local, national and international regulatory organisations and NGO's. We hold two fisheries steering group meetings a year where we disseminate our latest findings to a wide range of organisations. We organise conferences, workshops and events.

We will write several articles a year in our own and other stakeholder publications.

We will publish our work in peer reviewed scientific journals (The fisheries department published 29 papers in the last 5 years)

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, a fish will be captured from the wild (e.g. river) by electric fishing and immediately placed in a holding container with river water where they resume normal swimming behaviour within 2-5 seconds. The fish will be placed in anaesthetic for 2-3 mins until anaesthetised. Approximately 90% of the fish used under licence will be placed on a processing table to be weighed, measured, scale samples taken for ageing and genetics and then implanted with an internal PIT tag. This takes some 20 seconds per fish. PIT tag's are about the size of a grain of rice is implanted using an injector or minor incision of 2mm.

Approximately 10% of the animals used under licence will experience the procedures above and In addition, they will undertake surgery to implant a larger (circa 10-20 mm x 5-10 mm tag) into their body cavity. This involves making a incision of 10-30 mm and applying between 1 to 3 sutures

A fish is then placed in a recovery container of river water for up to 30 minutes before being released back into the wild where it was captured.

Some of these fish maybe recaptured. - upon recapture:-

In objective 1, some 10-15% of the 10,000 juvenile salmon and 3,000 juvenile trout that were anaesthetised, PIT tagged, weighed and measured, adipose clipped and scales were taken in September of each year will be re-captured the following spring as they migrate to sea as smolts. These will be recaptured in a rotary, screw fish trap, where they will be removed by hand net, anaesthetised, weighed, measured and the tag will be read with a PIT tag scanner. Once recovered, they are released back into the wild. On return to the REDACTED as adults their implanted PIT tags are read by automatic counters. Therefore, it is unlikely that these fish will be handled more than twice in their life.

In objective 2, the sea trout kelts, are captured by electric fishing in the winter, anaesthetised, weighed and measured, adipose clipped and scales taken. They will then



have acoustic and DST tags surgically implanted into the peritoneal cavity. Once fully recovered the fish are released. The following summer some 30% of these fish will return to the river where we aim to capture them in traps and by electric fishing. Upon capture they are killed using a schedule 1 technique. These fish will have undertaken the initial procedures when tagged and then killed when recaptured. These fish will be caught and handled at most twice in their lifetime.

In objective 2 we are undertaking long term studies on grayling populations. Here fish maybe captured and handled annually for the lifetime of the animal, at most 5 years. Similarly, in Objective 1 a proportion of the trout will not migrate to sea as sea trout but remain in the river as brown trout. Here these fish maybe caught annually for again up to 5 years or the lifetime of the fish. However, the numbers involved will be very low.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Impacts are described in the Action plan, despite repeated captures we recorded no effect on growth. Fish may experience stress at being held in the holding containers and short term pain during fin clipping and PIT tag insertion.

The most significant potential adverse effects come from the surgery to insert acoustic and DST tags. The incision is much larger and the length of surgery is longer at 2-3mins. Upon release back to the wild we want these fish to behave normally, therefore each fish is given the greatest of care to ensure the implantation process is smooth and as stress limiting as possible. We have refined our methods to use the minimum number of fish acceptable for an experimental purposes, use the smallest tags available, staff training and competency is paramount here to ensuring the procedure goes smoothly and limiting the negative impacts on each fish.

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

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

Approximately 90% of the fish we will use will experience a mild severity and 10% will experience a moderate severity.

What will happen to the animals at the end of the study?

- 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 aim of the project is to examine natural behavioural patterns of free-living wild fish. This cannot be carried out by any other means than using fish either from the wild or released into the wild from hatchery stock.



What was your strategy for searching for non-animal alternatives?

We have considered using existing or writing custom computer programs to create a realistic but a virtual population that is subjected to the experiment and then measured, which is repeated many times. The result is an estimate of the effect of the experiment on the virtual population and some measure of its uncertainty (and therefore statistical significance).

Why were they not suitable?

The aim of the project is to examine natural behavioural patterns of free-living wild fish, this cannot be achieved without using wild fish

Reduction

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

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

Numbers of animals that will be used are based on combinations of power analyses, statistical principles, ecological understanding derived from our REDACTED and other studies, and previous experience of logistical considerations. Our team has a wealth of experience and our proposed work is tailored to their abilities and limitations. All numbers and capabilities in the REDACTED have been reviewed and updated for this PPL.

REDACTED salmon population dynamics Originally, the numbers of animals needed to derive robust estimates for important population parameters, such as marine mortality rate, was based on power analysis. Today, our data suggest that the numbers of animals proposed by that original power analysis is required to ensure data are sufficient to draw robust inferences. Specifically, our original power analysis suggested that we needed to mark 10,000 salmon parr in autumn to recapture 500 as smolts in the following spring and to redetect 50 as adults returning in the 2 years subsequent to that spring. We marked 8,422 salmon parr in the autumn of 2015 that resulted in 245 salmon smolts recaptured in spring of 2016 and a total of 52 adults redetected over 2017 and 2018. Similarly, we marked 5,253 salmon parr in the autumn of 2016 that resulted in 158 salmon smolts recaptured in spring of 2017 and a total of 28 adults redetected over 2018 and 2019. The number of salmon parr tagged in 2016 and 2017 were lowest since 2005 due to low stock levels and the resultant smolt and adult data highlight the need for 10,000 fish to achieve the target number of recorded smolts and adults suggested by the power analysis. In 2018 and 2019 tagged the target 10,000 salmon parr and the data from the adults from these cohorts will be collected in the next couple of years. Ultimately, it is the numbers of redetected returning adults that limits the precision of our population parameter estimates, and the numbers recorded during the last PPL were sufficient, but not excessive, to derive meaningful precision, i.e., an uncertainty within 1 % of the estimated returning adult population abundance; many fewer individuals would have resulted in greater uncertainty and meaningless precision, i.e., an uncertainty between 0 – 100 %.



REDACTED trout population dynamics. To derive robust estimates for important population parameters, such as smolt contribution from different parts of the catchment and marine mortality of this partially migratory species fish have to be marked throughout the catchment in sufficient numbers to take account of high levels of natural mortality as well as part of the population remaining in freshwater all their life. We marked 2,848 trout parr in the autumn of 2015 that resulted in a total of 96 trout smolts detected in spring of 2016 and 2017 and a total of 24 adults redetected over 2017 and 2018. Similarly, we marked 2,736 trout parr in the autumn of 2016 which resulted in a total of 143 trout smolts detected in spring of 2017 and 2018 and a total of 38 adults redetected over 2018 and 2019. Tagging 3,000 trout parr annually provides us with the data that we need for the current analysis but we may be looking to expand this in the future to provide answers to further questions.

Salmon and sea trout smolt acoustic tracking In our REDACTED, we aimed to acoustically tag 60 salmon and 60 sea trout smolts each year in each river under study. This number was a balance between the number required to derive an accurate estuary survival rate estimate with meaningful precision, which has to account for factors such as the perceived average acoustic receiver efficiency at the planned locations, and the logistical constraints of the fieldwork, including factors such as staff time and budget. Of those 60 targeted salmon and sea trout smolts, we actually tagged 120 salmon and 120 sea trout smolts in 2017 and 120 salmon and 119 sea trout smolts in 2018. Preliminary analyses suggest that these numbers are sufficient, but not excessive, to derive meaningful precision.

Sea trout kelt DST tagging In our REDACTED, we aimed to acoustically tag 50 sea trout kelts each year in each river under study. As for the smolt acoustic tracking study, this number was a balance between statistical requirements and logistical constraints. Again, preliminary analyses suggest that these numbers are sufficient, but not excessive to derive meaningful precision.

REDACTED and trout population dynamics For this study, the number of animals used is determined by the survey method – known as depletion; to derive species-specific annual abundance estimates using depletion requires data representing the number of captured fish of each species from a minimum of three consecutive electrofishing surveys. Therefore the final number of grayling and trout used will be proportional (according to the electrofishing inefficiency) to the number of grayling and trout present at the site. For this study, we have and will continue to replicate the depletion electrofishing surveys at six sites because that is the minimum number of sites required to represent the river using statistical hierarchical structures.

Salmon metabolic rates In our REDACTED, ran a pilot study consisting of 24 fish at each of five sites, however to undertake a more robust analysis of the potential effect of individual metabolic rate on future migratory strategy a larger sample size is needed.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

All experimental and behavioural studies undergo a pre-assessment statistical power analysis to determine the numbers of experimental animals required to meet the objectives of the study.

What other measures apart from good experimental design will you use to minimise numbers?



When collecting samples for e.g. genetic analysis the samples can often be preserved for further analysis ensuring that the maximum scientific benefit from samples and in the medium to long term reducing the number of fish that need to be sampled in the wild.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The tags and marks that we use on fish, enables us to not only identify the fish we have used, but saves us from having to re-use the fish with multiple procedures tags and marks in the future. Therefore, these techniques refine the impact on fish over both the short and long term.

Methods used in this project will include:-

Fin clips removes the posterior third of a rayed fin, the fin normally clipped is the pelvic fin. However, in the case of the adipose fin which we use to identify all our tagged fish and to save the risk of them having repeat procedures, we remove the whole fin. The adipose is the smallest and least used by salmonid fish, given all other fins are actively used for swimming. Therefore we believe, removing the adipose, will having the least functional impact on the fish and cause least harm.

Scales and tissue samples. - This is necessary to estimate age, growth, genetic sexing, DNA and stable isotope analysis. To limit suffering we make sure we only take the minimum number necessary from each fish (scales grow back after removal) and use the clipped adipose fin for tissue samples, removing the need for further flesh removal.

PIT tags are small transponder tags. The tags have an indefinite life and therefore continue to give individual identity information over the whole life-time of the fish. The PIT tags we use are 12mm in length and thus very small. the small tag is used to limit disruption to the fish and also to reduce the need for invasive surgical procedure. The tags can be inserted using an injector needle or small 2-3mm incision into the body cavity.

Floy' or streamer type tags - These are external identifying tag and rely on a plastic 'thread' being inserted into the fish muscle (normally the dorsal muscle adjacent to the dorsal fin) and being held in position by a 'T' bar in the muscle. They are required so that our DST tags can be recovered if the fish are caught by anglers or in a fish trap. To limit stress on fish these tags are only used on larger fish, mainly adults greater than 33cm in length.

Dye marks - In case the Floy tag falls out of the adult fish, we will use a Panjet dye mark to mark the belly again so that anglers and trap operators can identify our tagged fish so that we can recover our DST tags. Panjet mark is not suitable for small fish which can cause internal damage. Therefore, again,we only use this mark on larger adult fish greater than 33cm.



Stomach flushing - We may also take stomach content samples to determine if the in-river habitat is providing the optimum conditions for growth and survival. This involves passing water down a tube into the fishes stomach so flushing the contents out through its mouth. To limit potential damage to fish and suffering, we use a soft tube, suitable for the size of fish and water pressure suitable for the size of fish.

Elastomer marks - These are thin treads of coloured liquid elastomer injected into the fish fins or as subcutaneous dots on pale coloured areas of the fish. Combinations of mark site and colours on individual fish can give a high number of batch marks. Adverse effects are minimal

Visual Implant (VI) tags are small (2 mm x 4 mm) pieces of plastic film that have a code printed on it. They are injected subcutaneously into areas of clear adipose tissue on the fish (often just posterior to the eye). Some inflammation can occur at the injection site but a skilled operator and good injector hygiene will minimise this.

Acoustic, radio and DST tags - These are necessary to be able to collect data from free swimming fish. Tags are inserted via surgery into the peritoneal cavity. To limit suffering we have refined our surgery procedure to include:- Ongoing training of suturing on dead fish, invested in suitable surgery equipment, ensure that equipment and surgery area is kept sanitised, accurate use of anaesthetic.

We constantly strive to refine our methodology to cause least harm, to ensure this only fully trained staff and those deemed competent will undertake this work. Our work is reviewed by our Vet twice a year with on site supervision and each person is approved for competency.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The fish named in this licence are the subject of interest and must be kept alive to be released for future studying. Therefore we cannot use any other animals or species or animals that have been terminally anaesthetised.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Myself and members of the fisheries team will keep up to date with current advances in scientific analysis and sampling techniques.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Electric fishing is one of the main techniques we use to capture fish. We have written manuals and guidance on best practice to maximise fishing efficiency but by immobilising fish in the most sensitive way to ensure they are in good condition for research and monitoring purposes. We review literature and work with manufacturers of fish tags to feed back information on the quality of data obtained and also the observed experience of tag insertion and impacts on fish.



How will you ensure you continue to use the most refined methods during the lifetime of this project?

Undertake module 5 training every 5 years. I will read and and distribute, information obtained on the 3RS from relevant websites, including the ASPA gov. We sometimes work in collaboration with other universities and research groups and during collaborations we discuss best practice for ASPA work. We constantly strive to improve our processes and experimental protocol.

Explain the choice of species and the related life stages

We are using salmon, trout and grayling because these are the fish of interest and it would not be possible to investigate our hypothesis using substitute fish or not using fish at all. We need to observe the natural behaviour of these animals in the wild. We will use adult trout, salmon and grayling and also juveniles called fry, parr and smolts. We will not use eggs. We will only use methods that are suitable for each life stage of fish, e.g. we will not insert acoustic or DST tags into 0+ fry.





70. Induction, assessment and prevention of adhesions

Project duration

1 years 0 months

Project purpose

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

Key words

No answer provided

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Post-surgical adhesions (PSAs) consist of fibrous tissue which sometimes grows excessively and can lead to constriction of the bowel and other internal structures, cause significant pain and even result in female sterility.

This licence will predominantly cover work done as part of our device development strategy and as such will focus on novel minimally invasive devices and new/modified techniques related to them. However, the intention is also to use this as a service licence to investigate other devices/ compounds with the ability to aid in the reduction of the problems associated with adhesions.

In a systematic review of 87 studies including 110,076 patients the incidence of small-bowel obstruction due to PSAs was 9% which is equal to 9,906 patients over a period of five years. If these figures are extrapolated to include adhesions at other sites (which have not yet been exposed to systematic review) it is likely that an excess of 10,000 patients per year could benefit from an effective PSA prevention strategy.

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



What are the potential benefits that will derive from this project?

The long-term benefit is the reduction or prevention of post surgical adhesions in both humans and animals will reduce post-operative complications, enable efficient recovery to normal movement, reduce or remove the need to carry out subsequent surgery to remove adhesions and thus improve patient welfare, reduce hospital in-patient time and reduce the financial implications.

The short-term benefits will be the development of new devices through initial in-vivo testing and the generation of data for submission to regulatory authorities.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Over the 6 months of this licence we would aim to use approximately 20 sheep and 60 pigs.

Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The models we use create adhesions, but we do not let these adhesions become painful to the animals as we treat just after creation to assess reduction or prevention and we know from previous studies that the treatments we use have good potential to reduce or prevent PSAs so we would regard this licence as only moderate in its severity. Some animals will be recovered from surgery and will be monitored for up to 6 months after the initial surgery. This may include repeated anaesthetics for the purposes of biopsy and/or non-invasive imaging. Any animals who show excessive signs of distress will be put down and examined in an attempt to determine the cause and also to assess the effect of the treatment applied to them. At the end of each study the animals will be put down and the tissue taken and examined to assess the efficacy of the treatment, also, where possible, tissue will be taken for other studies and/or educational purposes in an effort to maximise the usage and reduce overall number of animals used.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

The formation of adhesions is a complex process involving many different components within the body (blood, lymph, enzymes, etc) all interacting and as such a complete live animal is needed to form adhesions for evaluation and subsequent treatment. Prior to live animal studies, procedures, materials or devices to be assessed will, where possible, be tested on cells or tissues in order to keep animal use to a minimum.



Reduction

Explain how you will assure the use of minimum numbers of animals.

All potential treatments, procedures or devices transitioning from the laboratory into live animal testing will go via pilot studies involving small numbers (typically 3) of animals - this is to be sure that the laboratory prediction is borne out in live tissues.

For many of the studies carried out under these protocols, several sites of injury can be induced in the same animal which allows us to reduce the number of animals required to produce scientifically relevant data. Also, the ability to use adjacent or remote tissues from the same animal as internal or autologous controls again allows a reduction in the number of animals required overall.

For those studies carried out under Good Laboratory Practice (GLP) compliance, a regulatory process required by the Medicines and Healthcare products Regulatory Agency (MHRA) and the Food and Drug Administration (FDA) for all pre-clinical studies leading to requests for use in man, statistically robust appropriate information must be derived and this typically requires between 6 and 10 animals per experimental group to satisfy these parameters.

The ability to remove organs and tissues under terminal anaesthesia from animals in one study to be used for in vitro or ex vivo studies or transplantation/implantation or to be used for training reduces the need to retrieve these organs or tissues from dedicated donors thus reducing the number of animals required overall.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The models we use have been evolved over the last 20 years and are as refined as we can currently achieve. We use smaller species (mice and rats) for initial studies to confirm that the laboratory prediction is borne out in live tissues but often need to use more appropriately sized animals (i.e. a similar size to humans) for many studies. Using a range of assessments including non-invasive imaging (e.g. X-ray or Ultrasound) has further refined our techniques allowing us to obtain more information whilst minimising the impact on the animals' welfare.

For some direct application treatments, to establish representative sized defects and relevant treatment doses, large animals are required. Also, for the new procedures, instrumentation is designed for humans and a representatively sized animal will therefore have to be used. There are some areas of anatomy which are specifically recognised within different species as best models – e.g. for meniscal cartilage the sheep is deemed more anatomically similar to humans than is the pig, while for bowel and vasculature the pig is deemed more representative of the human than the sheep. Choices of species will be dependent on the anatomic site under investigation.

Appropriate monitoring of animals post-surgery and intervention if necessary with pain relief medication will ensure animal comfort. Our experience is that the animals are not in any pain during these studies probably because most are treated and those that are not



are not allowed to progress to the level of adhesion formation where humans would present with symptoms.



71. Intrinsic & extrinsic effects on B cell differentiation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

The project aims to define the extrinsic and intrinsic factors involved in B cell differentiation

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 produce data that increase our knowledge of how B cells respond to immunisation. B cells are lymphocytes that are best known for producing antibody in response to infection. The production of antibody and memory B cells is crucial to successful protective immunity and without B cells, humans and mice are severely immuno-suppressed. The function of vaccines is dependant upon the effective production of an antibody response and, as such, it is of crucial importance to understand how B cells respond to antigen and, furthermore, to improve upon our current therapies through increased knowledge of the basic mechanisms. B cells also can initiate immune responses through ferrying and presenting antigen and they have been shown to regulate immune responses in models of disease in mice such as EAE and collagen induced arthritis. The therapeutic benefit of depleting B cells in arthritis have indicated the regulatory roles of B cells in autoimmunity beyond autoantibody production, and understanding how B cells hyperactivated when cross-reacting with self antigens may lead to ideas how to treat autoimmunity and how to induce self-reactive responses therapeutically, e.g. in cancer



therapy. Our clinical work has shown that diurnal changes are important for the efficiency of vaccine responses, especially in older individuals. This study will identify diurnal changes in B lymphocytes causing this effect, which may lead to ideas on how to improve the antibody response to vaccination.

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

This project licence and the experiments described within it will produce data that increase our knowledge of how B cells respond to immunisation, specifically, to study how B cells undergo affinity maturation and generate memory to immunisation, and to study the signals regulating B cell differentiation in locally inflamed tissues. This will encompass both how B cells behave in isolation and how they interact with their environment (e.g. other immune cells in the local microenvironment).

The project represents basic research, and within the lifetime of the project it is unlikely that there will be direct benefit to patients. It is anticipated that this body of work will be published in high ranking international journals and discussed at scientific meetings. This work will give valuable information on the improvement of vaccine strategy on aged people, and our understanding of B cell autoimmunity.

Short term benefits will be advancements in basic science of B cell differentiation in response to infection and vaccination, the development and maintenance of humoral memory, and on the mechanisms of activation of self-reactive B lymphocytes.

Medium term benefits may be new ideas how vaccine responses can be enhanced, or how new vaccines to difficult to target antigens may be formulated, or how vaccines generating cross-reactive immunity may be generated, or how antibody responses in the aged immune system may be enhanced. There may be new ideas on how autoimmune B cells can be targeted therapeutically, or how ongoing autoimmunity may be targeted to slow the disease process. Further, this may lead to new ways of inducing autoreactive antibody responses, e.g. in cancer therapy. Lastly, the project could generate new mouse strains that are useful in the generation of new monoclonal antibody drugs for industry.

Long term benefits may be the translation of these ideas into clinically effective drugs.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The outputs of this project will have very broad reach, encompassing basic research that will inform the fields of immunology, vaccinology, and cancer immunotherapeutics, and at the same time improving vaccination on aged people, and developing a potential target on B cell in autoimmune disease, a broadly applicable new cancer vaccination method towards the clinic.

The project will have broad academic benefit, provide new knowledge on signals regulating GC B cells selection and antibody response (e.g. antibody feedback, circadian), and information on the role of polyclonal antibody responses to autoantigens by active vaccine and new ways of inducing autoantigen specific antibody responses. New reporter mice provide how to select high affinity GC derived plasma cells to supporting the development of high affinity hybridoma. new B cell anergic mouse model helps understand the phenotype of anergic B cells, and how to regulate anergic B cells to responding auto-antigens, and how immune tolerance broken down in autoimmune diseases. The work on



B cell anergy can produce data on immunology of autoreactive B cells after stimulation, which will crossfertilize other fields, e.g. HIV vaccine development, where neutralizing antibody is derived from anergic B cells.

The project has potential benefits on clinic. The research about the effect of circulation for vaccination has the strong benefits to improve vaccine strategy on aged people. the treatment of various solid tumours. This active vaccine to induce polyclonal antibodies to auto-antigens has the potential benefits to the treatment of various solid tumours.

The project also has potential commercial benefits. The improvement of vaccination on aged people could activate good antibody responses to vaccination and infection, which would benefit patients and the NHS. New reporter mice could improve the development of high affinity hybridoma, which has considerable potential effect on the monoclonal antibody production. Monoclonal antibody have been widely used on the basic research and clinic diagnosis, also have shown huge potential in cancer therapy. We are in the early phase of developing a cancer therapeutic vaccine by active vaccination to harness immune responses to auto-antigens in cancer. This vaccine strategy may be licensed and produced for the worldwide market for treatments of tumours, even potential benefit for the antibody mediated autoimmune diseases. This project has considerable potential to benefit the UK pharmaceutical industry.

How will you maximise the outputs of your work?

The output of project will be presented and discussed on national and international conferences. Outputs will be published in high quality open access journals and the UoB websites regularly updated. Manuscripts for publication will be prepared according to ARRIVE guidelines.

At this establishment we are in collaboration with colleagues who work on humans to study how B cell differentiation changes in the aged immune system, effect of circulation on antibody response, to improve the vaccine strategy on aged adults. We collaborate with industry to develop B cell reporter mice, and anergic B cell models. We start collaboration with colleagues who work on translational immunological research, and medical oncologists to address the B cell functions in autoimmune disease, cancer. We also collaborate with researchers from other institutes/Universities in UK or outside.

Species and numbers of animals expected to be used

- Mice: 30000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, mice will experience mild, transient pain and no lasting harm from immunisation by injection using standard routes or injection into the foot. Foot immunisation will be done under anaesthesia and lead to mild swelling that will not affect normal behaviour. Mice will be reimmunised or injected immunomodulatory substances. No animal will be injected



more than four times. Blood samples will be taken at the beginning of the experiments, before and after immunisation. Animals will be killed within 6 weeks of immunisation. The test substances will already have been tested to ensure that the dosing regimen does not cause toxicity. 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.

A significant proportion of mice will receive repeat immunisations into the foot under anaesthesia. Swelling after injection may result in mild pain, but should not result in obvious changes in behaviour.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Expected impacts are short-term stress and pain from animal handling and injections. Foot immunisation will often lead to foot swelling that can last a week.

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

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

We expect most animals to experience mild severity due to procedures such as ear-clipping, intraperitoneal or intravenous injections or taking of blood samples. 20% may experience moderate effects, mainly due to foot swelling after foot immunisation under anaesthesia, or occasionally when a larger number than three injections are necessary.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 antibodies are generated in response to vaccination. This process happens in lymphoid tissue that are very complex structures with many different cells types interacting and communicating with each other. In addition, these immune cells are in constant movement that allows them to interact with different partners at different stages of their development. These processes are so complex that currently no in vitro system is able to replicate this.

What was your strategy for searching for non-animal alternatives?

computer modelling of immune responses

In vitro models



Why were they not suitable?

Whilst computer modelling of immune responses is ongoing and improving these do not yet begin to replicate the complicated conditions and variables that exist in the immune system. Our use of computer models helps to confirm some of our data and provide testable hypothesis but is limited in scope and output to small aspects of the B cell response in vivo.

In vitro models are useful for very simple replication of immediate B cell stimulation and are used in our laboratory when appropriate but, again, do not replicate the immune system and the complexities of the environment in which immune responses occur.

Reduction

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

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

We have consulted a statistician for power 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, Power calculations using exemplary data from this study showed that group sizes of 8 are needed to achieve a power of 0.9 with alpha of 0.05 and effect size of 2. For experiments of central importance for the study we would work with alpha of 0.01, which would demand group sizes of 12. Typically, these group sizes are achieved by doing two independent repeat experiments. Most animals are generated during breeding and we have used historical data to estimate potential numbers.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Advice from local professional statisticians has been sought to evaluate proposed experiments for statistical validity and in the generation of power calculations. Numbers of animals necessary were calculated using our own historical data to estimate the expected variability of our experiments and to calculate the minimal number of animals necessary to generate significant results.

Further, we are continuously refining our analytical methods in order to reduce variability, allowing further reduction in animal numbers. All relevant tissues where possible will be frozen and stored as input and controls for downstream experiments. Shared use of these will be offered for shared use by other groups working on related questions.

What other measures apart from good experimental design will you use to minimise numbers?

We will minimise use of animals by keeping colony sizes as small as possible. This may lead to experiments having to be split into two independent repeat experiments in order to generate sufficient power. New substances used on animals will be tested first in small pilot studies. Computer modelling may be used to predict experimental conditions that will



show the largest effect sizes. At the end of the experiment we will harvest the maximal possible number of tissues. Tissues not immediately analysed will be archived frozen and will be made available to other researchers working on similar questions.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Some animals will be allowed to age in order to study the immune response in the ageing organism.

In order to induce gene expression in animals or deplete specific cells some animals will have receive substances via gavage, injection, or through food. Oral gavage or injection can be necessary in order to induce a rapid onset of gene expression. This will allow us to study processes that happen within short time periods of a few hours.

We will have to induce immune responses in order to study the response to vaccination. Animals will be vaccinated using methods similar to human vaccination, e.g. injection of substances under the skin, or by intraperitoneal or intravenous injection.

Some animal will have to be vaccinated into the foot, as this induces a strong response in local lymph nodes. This will be done under short term anaesthesia, however, the animals will suffer from temporary swollen foot for several days. This has not led to changes in normal behaviour in the past indicating that there is no major discomfort. If animals do show signs of behavioural change during the foot swelling phase, they will be treated with analgesic agents.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Non-mammalian animals are limited in their use because they either do not possess B lymphocytes or their differentiation is too removed from the human immune system to provide relevant results. Embryos are unsuitable as their immune system is not mature and does not respond to antigenic stimulation in the way mature animals do.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to 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 due to old age. Longer drinking spouts will be used, and animals will be monitored for adverse effects such as changes in weight, dermatitis, piloerection, paleness, changes in mobility, lumps, eye defects, abnormal



respiration or stools. If these are observed animals will be treated accordingly, and animals with that may develop severe effects will be killed humanely.

If gene induction or deletion is done for a new gene animals will be monitored closely in the days after induction. Mice will be weighed on a daily to detect weight loss. If any mice have reduced activity, ruffled coat or hunched appearance they will be warmed and given glucose-saline (sub cut) to reduce heat loss and dehydration which may be a contributor. Flexible gavage tubes dipped in sucrose will be used to minimise damage to the oesophagus. Good handling and technical expertise will minimise any discomfort. Time and route of induction will be optimised in preliminary experiments for efficient induction of deletion of transgenes using the least adverse route of administration.

For all new models, new methods and new antigens we will consult with expert staff at our animal facility. Some methods, e.g. animals that are receiving new antigens or immunisation protocols, will be carefully monitored during the protocol and humane end points will be used if necessary in consultation with expert staff at the animal facility.

Immunisation will be done via injection of antigen. These vaccinations should only have transient effects and animals should return to normal behaviour within two hours. If new antigens will be tested, this will be discussed with expert staff at the animal unit. New antigens will be tested on small groups of animals first.

Some animals are immunised in the foot under short term anaesthesia. Foot immunisation may lead to foot swelling due to inflammation. This should not be strong enough to lead to behavioural changes. Mice that do show excessive inflammation or lameness will be treated with analgesics. Good handling and technical expertise will minimise any discomfort. Mice are not re-injected until fully recovered from previous injection and never at a frequently that causes them to display anything other than transient pain to discomfort. Foot immunisation has been refined by injecting substances under the plantar surface of the foot, away from the weight bearing walking pads. Further, injection of non-immunogenic substances will be done by hock injection.

LASA guideline will be followed regarding volume of substances to be administered.

Pathogens used are weakened or killed versions that should be non-pathogenic within the time-scale of the experiment. When pathogens are used, animals will be monitored appropriately. In the case of bacterial infection mice will be monitored for the first 48 hours following immunisation and also during the third week of infection which is the time of high susceptibility of secondary infection. The dose of bacteria administered is the lowest possible to obtain a response. Expert animal handling staff are aware of the course of the infection and adverse effects are expected to be noticed and dealt with quickly.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Experiments will be performed in line with LASA guidelines.

Will ensure that all experiments are designed to allow reporting in accordance with the ARRIVE guidelines. Our research will be published according to ARRIVE guidelines, regardless of whether a journal endorses this.

How will you ensure you continue to use the most refined methods during the lifetime of this project?



We will regularly check information on NC3Rs, have signed up to the NC3R newsletter, and will meet with the NC3Rs Regional Programme Manager, and attend Regional 3Rs symposia.

Explain the choice of species and the related life stages

We are using adult mice, because they are the only well studied mammal that has an immune system that is sufficiently similar to the human immune system. Only in mice have experimental methods been developed that will allow us to undertake our work.





72. Investigating novel formulations & the delivery of vaccine and drugs

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

This research work aims to provide new formulations that may improve efficacy of already available therapeutic agents or vaccines, to facilitate the formulation of substances that might otherwise be inactive, and improve storage characteristics (e.g. increased shelf life, or heat-stability, thereby avoiding the need for cold-chain vaccine handling).

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

Why is it important to undertake this work?

Identification and characterisation of drug and vaccine formulations and delivery systems that improve stability or biological action could lead to significant benefits in terms of contribution to healthcare quality worldwide. In real terms, we would expect that work done



under this project will help to outline facets – such as techniques and the use of novel agents – of drug and vaccine delivery systems, that may provide the basis for further research and possible clinical trials. The benefits may include the dissemination of research from this project that results in the adoption of more effective formulation strategies – the development of knowledge through this project may lead to implementation of improved formulation strategies within the pharmaceutical area by publishing results based on these studies.

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

Identification and characterisation of drug and vaccine formulations of interest and delivery systems that improve stability or biological action.

We would expect a similar output to the previous Licence. It is hoped that specific candidate drugs, vaccines and formulations of interest that have been studied through the project may enter pre-clinical and clinical trials or go on to be manufactured to benefit the population.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The work has the potential for widespread improvement of human health, depending on the specific candidates analysed.

There remains a clinical need for safer and more effective medicines and vaccines, this project could lead to significant benefits in terms of contribution to healthcare quality worldwide both for animals (e.g. TB and Rabies vaccines) and humans (e.g. TB, RSV and flu vaccines).

How will you maximise the outputs of your work?

Dissemination will be via delivery at conferences and peer reviewed publications.

We have several collaborators around the UK and of course all funders will also disseminate the outputs from the project.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 200

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

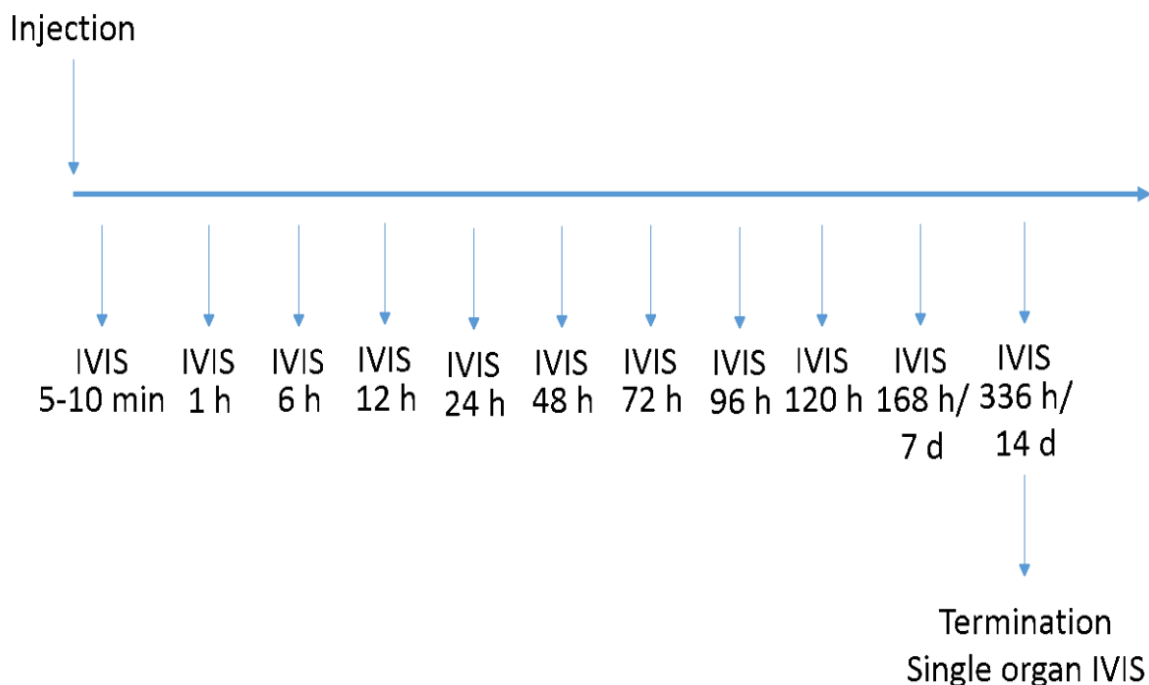
For protocol 1 animals will be administered substances (including radioisotopes) with or without anaesthesia, the route would be in the form of an injection (S/C, IM, IP, ID,IV),



inhalation, intranasal dosing or oral gavage three times over the course of an experiment, on a once every two week schedule, this would be interspersed with taking blood samples 4 times (one before the first dose and then in the opposite weeks from the dosing). Animals will then be culled using cardiac punctures to obtain a good volume of blood for the study or a schedule 1 method will be used.

Schedules will involve inoculations no more than twice in any one week and no more than six inoculations within the duration of the procedure; and no more than three times in any one week and no more than seven oral inoculations within the duration of the procedure.

For protocol 2 animals will be imaged using the IVIS which requires them to be administered fluorescently labelled substances, this could be done without anaesthesia, by one or more of the following routes: Intraperitoneal, intramuscular (normally into the quadriceps), subcutaneous (normally into the scruff or at the lower back), oral (gavage), intravenous. Optionally, a suitable dye (e.g. pontamine blue) is administered by s/c. Alternatively, under general anaesthesia, substances may be administered via one of the following routes: Instillation by the intranasal and inhaled route, intradermal injection or intratracheal gavage. IVIS non-invasive imaging involves anaesthetized animals being placed inside the IVIS imager for a limited time and then allowed to recover. Animals may occasionally be imaged for up to one hour, but typically for less than 15 minutes. Animals will be allowed to rest for a period of no less than 1 hour between imaging sessions. Any one animal may be imaged up to 15 times, with no more than 4 imaging sessions in one day (with the exception of pilot studies). A study may last 1 month, with imaging sessions spaced throughout. During a pilot study this may be increased as shown below:



Chemiluminescent substances (e.g. Luminol) reacting with reactive oxygen species (ROS) may be administered to the animals via the i.p. route prior to imaging (10-20 minutes). The animals will be imaged using IVIS as described in step. 3 above. Animals may be inoculated up to two times with fluorescently labelled substance, though typically one inoculation will suffice. The inoculations will be at least two days apart.



Animals will then be culled to obtain a good volume of blood for the study or a schedule 1 method will be used.

Below shows the information within the establishments dosing guidelines for each likely procedure listed there, these will be the guidelines followed. Administration of drugs/substances: to reduce discomfort as much as possible, volumes administered will not exceed the recommended maximum volumes above. If there is a justified need for larger volumes, the Home Office will be consulted. The least invasive routes (i.e. oral in water or food and subcutaneous) will be used in preference to the other routes whenever possible.

General anaesthesia: momentary discomfort/fear; minimised by good technique. Risk of anaesthetic deaths estimated at less than 1%.

Intramuscular injections: can be painful in most species. This route will be avoided whenever possible and the subcutaneous route used instead. If the intramuscular route is used, injections will be administered into alternate sites and with the minimum volume possible. Any animal showing persistent lameness due to muscle damage will be withdrawn from study or will receive veterinary attention.

Intraperitoneal injections: momentary discomfort. There is a risk of damage to internal organs and peritonitis (rare event). The risk will be minimised by good technique. Any animal showing possible signs of peritonitis (weight loss, hunched posture, piloerection) will receive prompt veterinary attention or will be euthanased.

Subcutaneous injections: momentary discomfort; minimised by good technique. Very rarely, swelling and/or erythema. If these develop, or if there is skin ulceration, veterinary advice will be sought.

Intravenous injections: momentary discomfort. There is always a risk of haemorrhage, phlebitis, haematoma or thrombus formation (rare events). These risks will be minimised by good technique and by applying localised pressure to minimise swelling and bleeding. If any animal develops adverse effects relating to venous sampling, it will be withdrawn from study and brought to the attention of animal care staff.

Gavage: momentary discomfort. There is also a risk of oesophageal damage and/or inspiration of gavaged substances (rare events). This will be minimised by good technique. Any animal showing possible oesophageal damage (neck swelling, weight loss, hunched posture, piloerection, excess salivation) or aspiration of contents (acute dyspnoea) will be humanely killed.

Intradermal injections: can be painful. They will normally be carried out under general anaesthesia or sedation. Local erythema and swelling are commonly seen; ulceration is a possibility. If present, ulceration normally resolves within 1 week following onset of symptoms, without the need for treatment. If there are no signs of healing after 3-4 days or if there is secondary bacterial contamination or if the animal shows signs of pain or distress (e.g., scratching/licking), it will euthanased or will receive prompt veterinary attention. Good technique will be used and close observation following injection.

Intranasal dosing: short lived nasal irritation and mild increase in respiratory rate. Very rarely, severe dyspnoea. If any animal develops severe dyspnoea, it will be immediately killed.



All blood sampling will be done based on the establishments good practice guidelines. The guideline can be accessed in the establishment. If bleeding cannot be arrested or there is obvious bruising causing irritation e.g. shown by the animal licking or rubbing the site or there is an obvious haematoma a veterinary surgeon will be called.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Protocol 1:

Drug Administration: There are no adverse effects anticipated as a result of the drug delivery systems administered. However, when working for the first time with systems with unknown adverse effects, initial studies will be conducted using low doses and one mouse at a time to ensure there is no related toxicity issues. To reduce discomfort as much as possible, volumes administered will not exceed the recommended volumes stated in the establishments dosing guidelines. The most appropriate administration route will be chosen, taking into consideration the administered substance.

Animals will not be allowed to lose more than 10% weight loss compared to an aged matched control. Any animal exhibiting either partial piloerection / subdued behaviour / hunched posture / Oculo- nasal discharge for longer than 12 hours after injection will be humanely killed. Advice will be sought from the NACWO & NVS for any animal giving cause for concern.

Blood Sampling: Establishments good practice guidelines will be adhered to. The guideline can be accessed in the establishment. If bleeding cannot be arrested or there is obvious bruising causing irritation e.g. shown by the animal licking or rubbing the site or there is an obvious haematoma a veterinary surgeon will be called.

Radioisotopes: These will be administered at levels that are below therapeutic levels, and in our experience over 10 years appear to cause no adverse effects to the animals.

Mice injected with pontamine blue get a bluish tinge in the skin, especially at the nose, ears and paws. However, no adverse effects to health and behaviour in mice has been observed when applying pontamine blue.

How the adverse effect will be recognised: Animals will be monitored for the above listed adverse incidents. If there are any doubts as to the severity of the clinical signs of animals undergoing procedures, the named persons will be consulted and advice taken as to whether or not the end-points as authorised in the PPL have been reached. In all cases, where adverse effects occur appropriate measures will be taken to minimise suffering, for example saline may be administered in order to reduce the effects of dehydration.

Anaesthesia: The level of anaesthesia will be maintained at sufficient depth for the animal to feel no pain. Deaths resulting from general anaesthesia are most uncommon in our experience (<1%) and will be kept to a minimum by ensuring accurate dosing during gaseous anaesthesia. The depth of anaesthesia will be monitored using indices such as the pedal reflex, corneal response and depth of breathing.



Intranasal dosing: Animals will be monitored closely following light anaesthesia for intranasal dosing to ensure a swift recovery. Following recovery, animals will be monitored for potential side effects.

Animals whose clinical signs exceed or are likely to exceed mild severity will be humanely killed by a Schedule 1 method

Protocol 2:

Drug Administration: As above with protocol 1

Whole Body Imaging: this is not an invasive procedure but animals need to be still during the scanning session and therefore an anaesthetic is required. During scanning, animals will be kept warm by means of a heated platform. Repeated general anaesthesia is stressful to the animal, therefore, the number and length of imaging sessions will be kept to the minimum possible to answer the specific experimental questions. Similarly, the longest possible interval between anaesthetics (to allow best recovery) will be used, depending on the experiment. Prolonged and/or frequent anaesthetics may result in dehydration. If necessary, subcutaneous fluids will be given to the animals in consultation with the NVS. Any animal showing signs of uncontrolled stress (e.g. losing body weight), or any other sign of ill health, will be brought to the attention of animal care staff or will be euthanized.

Any one animal may be imaged up to 15 times, with no more than 4 imaging sessions in one day. In pilot studies, imaging will be done more often to establish the window of interest (but not more than 15 times in total), which depends on the administration route and administered substance. Once this has been established, in subsequent studies, the number of imaging session will be reduced to the maximum of 4/day. A typical study may last 1 month, with imaging sessions spaced throughout.

If a study requires 2 to 4 IVIS sessions on the same day, the mice will be treated to e.g. Nutella after the imaging session as a means of positive reinforcement to minimize the stress from the anaesthetic procedure.

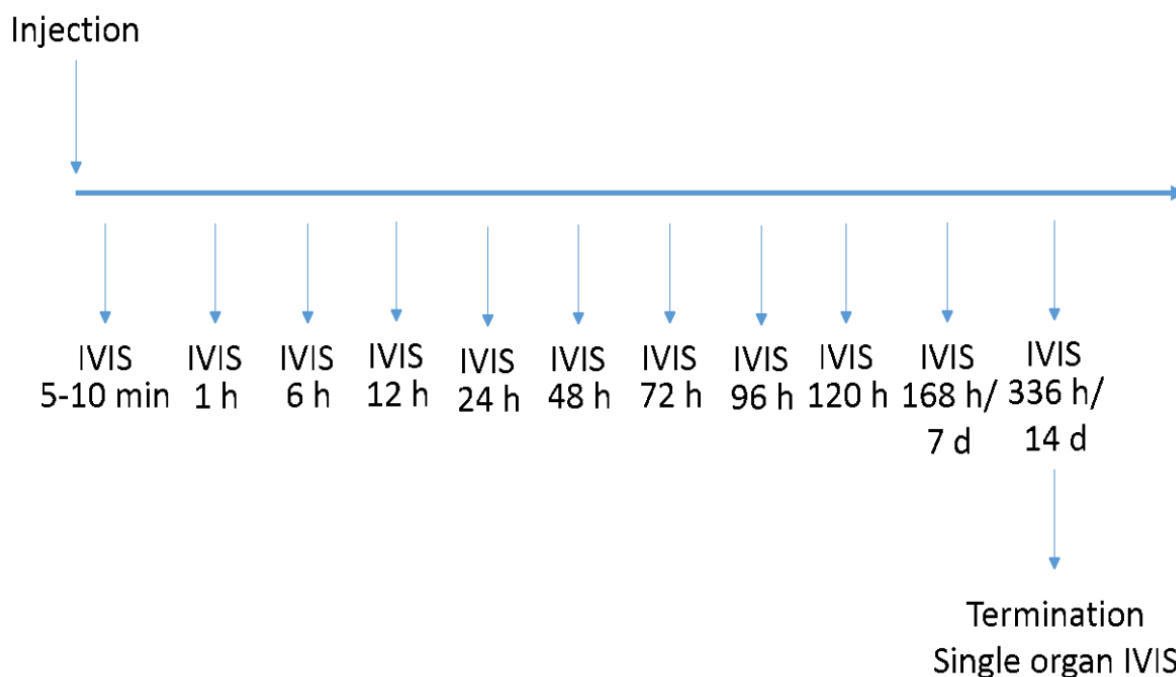




Fig. 1: Example of a study with one injection of fluorescently labelled substance followed by multiple imaging sessions. This is an example of a pilot study with many imaging sessions, fewer imaging sessions will be used in subsequent studies.

Anaesthesia: The level of anaesthesia will be maintained at sufficient depth to achieve light general anaesthesia. Deaths resulting from general anaesthesia are most uncommon in our experience (<1%) and will be kept to a minimum by ensuring accurate dosing during gaseous anaesthesia. Prior to entry into the IVIS, the depth of anaesthesia will be monitored using indices such as the pedal reflex, corneal response and depth of breathing. During imaging in the IVIS, the animal is monitored via the screen, anaesthesia is monitored by observing body movement.

Intranasal dosing: As above in protocol 1.

Blood Sampling: As above in protocol 1.

How the adverse effect will be recognised: Animals will be monitored for the above listed adverse incidents. If there are any doubts as to the severity of the clinical signs of animals undergoing procedures, the named persons will be consulted and advice taken as to whether or not the end-points as authorised in the PPL have been reached. In all cases, where adverse effects occur appropriate measures will be taken to minimise suffering, for example saline may be administered in order to reduce the effects of dehydration.

Animals whose clinical signs exceed or are likely to exceed moderate severity will be humanely killed by a Schedule 1 method. Animals will not be allowed to lose more than 15% weight compared to an aged matched control.

Any animal exhibiting either piloerection / subdued behaviour / hunched posture / Oculo-nasal discharge for longer than 12 hours after injection 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 species)?

Given there are no prospective adverse effects above mild for protocol 1 we expect that all animals would reach their stated severity.

Protocol 2 is assigned a moderate severity due to the possibility of up to 15 repeat anaesthesia sessions. This number will probably only be used at the start of tracking each substance. Therefore we would assess only 50 will be moderate.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 systems and physiology of lower vertebrates are significantly different from humans, including restricted antibody diversity (Roman et al. 1995) that may be partly due to the fact that some immunoglobulin segmental elements do not undergo genetic rearrangement (for example in fish [Litman et al., 1999]) in the same way as that which is seen in humans (and that also seen in mice). In addition, for biodistribution studies and drug delivery, the murine model may be the model of choice for these studies as the mouse physiology, in terms of organs and systems, has many parallels to that of human systems.

What was your strategy for searching for non-animal alternatives?

In conjunction with our in vivo studies, we have used these to develop of an in vitro testing model.

REDACTED

of in vivo liposome efficacy'. In these studies we have quantified liposome uptake by phagocytes using the human continuous cell line THP-1. Fluorescence-labelled liposomes were co-cultured with THP-1- derived macrophages at a final lipid concentration of 5 µg/mL. The proportion of macrophages associated with fluorescent liposomes, and the relative amount of fluorescence associated, was quantified using flow cytometry. Our results shows the time-dependent uptake of liposomes after application to THP-derived macrophages at 37°C; with changes in formulation influencing cellular association in line with in vivo results we previously found. We are continuing with these studies and using previously published in vivo data to back-correlate with the in vitro studies with the aim of validating this in vitro tool.

REDACTED

Why were they not suitable?

At present it is impossible to recreate the biological environment in terms of biodistribution throughout complex physiological systems following the administration of a therapeutic or prophylactic agent. For this and the evaluation of biological effects in some systems (such as the immune system mentioned here) animal testing may be 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?

This has been estimated based on our experience of screening formulations. Normally we will start this a high-through put model where we screen up to 20 formulations for the physical attributes. Form this at least 10 of these will progress in vitro and we then down-select a maximum of 4 formulations to test and include 2 control groups. This gives us 30 to 36 animals split over two studies (to provide intra and interday variability). We would



normally conduct 5 to 6 of these studies in rats over the course of the project (covering protocol 1 and 2).

For mice we use a similar design, however we have large research programmes working on vaccines. Normally we would conduct up to 12 to 14 of these studies per year (2 to 3 per researcher per year) (covering protocol 1 and 2).

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

In these and larger studies, good experimental design will ensure the combination of control groups and the comparison of multiple experimental entities or doses (or where appropriate, the utilisation of factorial experimental design).

The work conducted within these studies is based on improvement of formulations therefore control groups will be restricted to benchmark groups so that improvements can be tested and negative controls will be avoided unless absolutely necessary. All studies will be built on pilot data from either small group trials (n of 1 to 3) or based on previous data generated.

Advice on group numbers with reference to the data specific to our experiments will be obtained from the statistician responsible for advising the bioethical committee, who is experienced in statistics relating to animal experiments and peer review of proposed animal work.

What other measures apart from good experimental design will you use to minimise numbers?

IVIS is an alternative to performing biodistribution studies using radiolabelling of the administered substance. Because the imaging is performed under anaesthesia, the same animal can be used throughout the study, rather than having to euthanize animals at each analysis time point. In the long run, this can significantly reduce the number of animals used.

We will also only use animals when the lead compound shows activity in in-vitro tests.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Largely for reasons mentioned under 'Replacement' above, the mouse model is the most widely used model for initial vaccine immunogenicity evaluation and the initial in vivo assessment of developmental new drugs and delivery systems or formulations. The use of the mouse model enables cross comparison with other studies and hence more widely applicable results and context within which such results can be evaluated. We will draw on



previous experience and the latest research to ensure doses of antigens and adjuvants are correct and we will set the earliest possible end point for the experiments. All protocols will be performed under the mild/moderate prospective severity limit and animals will be monitored to ensure they do not go beyond this point.

Similarly, in terms of drug delivery and biodistribution, a large number of studies have utilised the mouse and rat models and evaluation and comparison of the results across the literature is possible. The rat model may be appropriate only where it is deemed that the murine model may not generate sufficient data (e.g. in some biodistribution studies) or where there is a need for cross comparison with other studies.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The immune systems and physiology of lower vertebrates are significantly different from humans, including restricted antibody diversity (Roman et al., 1995) that may be partly due to the fact that some immunoglobulin segmental elements do not undergo genetic rearrangement (for example in fish [Litman et al., 1999]) in the same way as that which is seen in humans (and that also seen in mice). In addition, for biodistribution studies and drug delivery, the murine model may be the model of choice for these studies as the mouse physiology, in terms of organs and systems, has many parallels to that of human systems.

At present it is impossible to recreate the biological environment in terms of biodistribution throughout complex physiological systems following the administration of a therapeutic or prophylactic agent. For this and the evaluation of biological effects in some systems (such as the immune system mentioned here) rodent testing may be necessary.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Biodistribution studies using radiolabelled substances will focus on endpoint experimental data and will thereby be restricted to mild severity unlike some studies where prolonged procedures may lead to moderate severity.

Biodistribution studies using IVIS require several imaging sessions with anaesthesia, and therefore these studies are considered of moderate severity. These animals will be provided treats (nutella or similar) after imaging sessions to help with recovery and so they also have some form of positive experience from the procedure.

Based on previous publications, we will not use Freud's complete adjuvant as a vaccine adjuvant control due to its potential for causing distress to animals.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The establishments good practice guidelines and the NC3Rs website. Also by ensuring that all training records are up to date and that all procedures are done by a competent person.

How will you ensure you continue to use the most refined methods during the lifetime of this project?



By checking in on the NC3R's website I can see of any new information from the source. As well as this, keeping up to date with current research advances could result in and of the 3Rs being improved. Also by raising any issues that arise with the NACWO, NVS and the technical staff with the aim to refine techniques to eradicate these problems.

Explain the choice of species and the related life stages

The immune systems and physiology of lower vertebrates are significantly different from humans, including restricted antibody diversity (Roman et al., 1995) that may be partly due to the fact that some immunoglobulin segmental elements do not undergo genetic rearrangement (for example in fish [Litman et al., 1999]) in the same way as that which is seen in humans (and that also seen in mice).

For biodistribution studies and drug delivery the murine model may be the model of choice for these studies as the mouse physiology, in terms of organs and systems, has many parallels to that of human systems. At present it is impossible to recreate the biological environment in-vitro in terms of biodistribution throughout complex physiological systems following the administration of a therapeutic or prophylactic agent. For this and the evaluation of biological effects in some systems (such as the immune system mentioned here) animal testing would be necessary.



73. Investigating post-partum welfare of cows and calves

Project duration

2 years 0 months

Project purpose

- 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

Cow, calf, welfare, behaviour, parturition, NSAID

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The welfare of farmed animals is under increasing scrutiny from consumers as well as governmental and industry bodies. Millions of cattle experience parturition (giving birth) every year worldwide every year and although parturition is a necessary event for cows, research into the welfare effects of parturition and birth are lacking. Most parturition in cows proceeds without the need for human assistance, however assistance is required in a substantial minority of cases each year. The size of this minority has been reported be up to 50% by some authors, which for the UK dairy industry alone equates to up to 900,000 parturition events every year. There is some evidence that assistance at parturition is more stressful and more painful for cows than unassisted parturition however studies researching the effect of administration of pain relief are limited. There is a paucity of research studying the effect of assistance on calves and it is uncertain whether assistance at birth is painful for calves and whether administration of analgesia improves calf welfare. There is some evidence that calves born to assisted births have subsequently poorer health and production than their counterparts born to unassisted parturition. The aim of this work is to build on previous work to further determine the welfare and health benefits for dairy cows and calves of administration of pain relief immediately following parturition/birth, and whether animals experiencing assisted calving receive additional benefits.

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



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

What are the potential benefits that will derive from this project?

The project will add to the current, limited knowledge base regarding any pain experienced and the welfare effects of parturition and birth in dairy cattle. The inclusion of analgesic intervention in the study will enable assessment of the welfare benefits pain relief to cows and calves in the post-partum period and has the potential to have far-reaching effects in improving post-partum welfare in cattle and calves. If pain relief is shown to be effective, it could be routinely administered by farmers to achieve significant welfare benefits for large numbers of animals.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

We expect to use up to 300 calves and 300 cows over the course of the project.

Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The severity level of this project is mild and serious adverse effects are not expected. Animals will receive an injection of pain relief or placebo. Some animals may experience localised muscle trauma at the site of intramuscular injection but the risk of this will be minimised by experienced personnel, appropriate restraint of the animals and correct volumes. Animals will be commercial dairy cows and calves, reared on a farm in the usual way, and following veterinary checks, they will be returned to the commercial herd after the study has ended.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

There are no alternatives to the use of cattle for this project because we are assessing welfare in this species. It is not possible to replicate parturition and birth in an *in vitro* environment.

Reduction

Explain how you will assure the use of minimum numbers of animals.

We have carefully calculated the minimum meaningful numbers of animals required, based on previous studies, and this will not exceed 300 cows and 300 calves. A factorial design



will be used to maximise statistical power and allow identification of interactions between our measures and causal factors, preventing the need for repeat studies and thereby minimising animal numbers.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Our use of cattle is essential as we are investigating the welfare of this species. We have minimised the welfare costs to the animals by using a pain reliever known to be well tolerated in this species and by it being administered by experienced personnel. The severity of the project is mild and severe adverse events are not expected; however if unexpected severe adverse events do occur, veterinary attention will be sought immediately and appropriate action taken under direction of the attendant veterinary surgeon.





74. Investigating the effects of sex hormones on heart disease progression

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Guinea pigs	adult

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The project has two main aims.

Aim 1: to determine the sex differences in the cardiac responses to high blood pressure.

Aim 2: to determine the mechanisms whereby oestrogens and related compounds affect heart structure and function in health and in disease progression.

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

Why is it important to undertake this work?

Heart failure (HF) can be considered to be the common endpoint of most types of cardiovascular diseases. As treatments for acute heart disease have improved, HF is



becoming increasingly common. There are sex differences in the response of the heart and cardiovascular system during disease progression towards HF, particularly following menopause in women. The underlying reasons for differences in this "remodelling" response have escaped rigorous investigation with an assumption that treatments for high blood pressure and HF are equally effective in both sexes. This work will determine the sex differences in the responses of the heart to maintained high blood pressure, the effect of sex hormones on these responses and examine if established HF therapies benefit both sexes equally.

The stimulation of certain oestrogen receptors in the membranes of heart muscle cells leads to an improvement in the relaxation of the heart and decreases the chances of arrhythmias occurring. The actions of sex steroids on the heart are largely unknown and while the past notion that "oestrogens are good" is too simplistic, new evidence indicates their capacity for benefit in specific situations. The work in this part of the project will examine the effects of sex hormones on the heart.

This will result in knowledge necessary to develop ways of moderating disease progression and improving current treatment strategies and may have considerable implications for the optimal instigation of such treatments thereby allowing a more logical approach to their use.

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

Heart failure (HF) can be considered to be the common endpoint of most types of cardiovascular diseases. As treatments for acute heart disease have improved, HF is becoming increasingly common. We know some of the disease outcomes at the molecular level but we do not know the effects of progression of the disease on the function of key proteins involved in the electrical and mechanical functions of the heart. This work will characterise these.

Although sex differences in the remodelling of the heart in the progression towards HF have been acknowledged, the underlying reasons have escaped rigorous investigation with an assumption that treatments for hypertension and HF are equally effective in both sexes. This work will determine the sex differences in the cardiac responses to a high blood pressure and examine if established therapies benefit both sexes equally.

The activation of certain oestrogen receptors improves the relaxation of the heart (so it fills with blood more easily) and decreases the likelihood of arrhythmias occurring. It is important to determine the mechanisms whereby sex steroids affect the anatomical and physiological changes that take place in the heart during progression towards HF. This will result in knowledge necessary to develop ways of moderating disease progression and improving current treatment strategies.

The results of this work will be presented at scientific meetings and will be published in peer-reviewed journals to ensure wide dissemination to the appropriate audience.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

This work will lead to a better understanding of the mechanisms involved in the different responses of the two sexes to HF. At the level of the cardiac muscle cell this type of investigation has not been done before. Other REDACTEDs at our establishment will



benefit from this knowledge and with publication and conference presentations, other groups in Europe and elsewhere. As a clearer picture of sex differences in the progression of heart failure emerges over the duration of the licence, interest from the pharmaceutical industry is likely because the results may influence a more tailored – and therefore more effective – approach to treatment strategies that may benefit both sexes.

How will you maximise the outputs of your work?

The results of this work will be presented at scientific meetings, will be published in peer-reviewed journals to ensure wide dissemination to the appropriate audience and these information routes will encourage further collaborations and potentially lead to translational opportunities.

Species and numbers of animals expected to be used

Guinea pigs: 650

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will undergo constriction of the main artery that leaves the heart and this induces a pressure overload on the heart that mimics high blood pressure. The hearts undergo a gradual increase in growth of muscle cells, changes in their electrical and contractile characteristics and progress to failure and this takes about 150 days. The surgery is carried out under aseptic conditions and uses effective anaesthesia, analgesia and antimicrobial and fluid replacement treatments. Non-invasive ultrasound scans will be made on selected groups of animals after the post-operative recovery until the end of their study time to assess heart size and function.

Female animals may also undergo surgery to remove both ovaries which mimics the menopause. Drugs that are used therapeutically to reduce blood pressure and stimulate or inhibit oestrogen receptors may be administered to assess their effects during the progression to heart failure. The animals will be killed humanely and the hearts removed. From these we will isolate single heart muscle cells, or slices of heart tissue or use the whole heart. The cells or tissues are then subjected to a variety of electrophysiological, fluorescence and molecular techniques.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Short term impacts

As with all invasive procedures, there is risk of operator error to cause trauma or even possible death. However, this procedure has been carried out successfully for over 20 years, with minimal (approx 4%) mortality due to surgical or anaesthetic error *per se*. The



small number of animals affected will not suffer any distress because they will be fully anaesthetised.

A number of animals (<15%) could develop acute heart failure (occurring between 6 – 24 hrs of the aortic constriction) if they fail to adapt to the constriction. This is the most common cause of peri- operative death and is more common in females than males. It is unpredictable in nature and preceding clinical signs are difficult to recognise over and above usual post-operative somnolence or drowsiness. Analgesia levels are high in the few days following surgery so animals will not experience pain.

Some animals can scratch or pull sutures apart before the wound has healed and if this happens wounds will be cleaned and re-closed.

Most animals have an uneventful recovery and will be housed in groups to encourage social integration. Together with regular gentle handling and cage enrichment these measures minimise distress and alleviate boredom during the long-term studies.

Longer term impacts

In the longer term (>7 days after the constriction operation) there is myocardial remodelling that can give rise to disturbances in electrical impulse conduction and propagation, and to the mechanical cycle of contraction and relaxation. There is a risk that a small number of animals may die from sudden cardiac death that, like in the acute cases, is probably caused by lethal arrhythmias but the exact cause of death is uncertain. Such sudden death during the progression towards HF is a relatively rare event (approx 2 %), but is unpredictable in nature because there are rarely any preceding clinical signs, akin to the human situation.

In humans, oestrogen deficiency is a cause of bone loss occurring during the first two decades after natural menopause. Ovariectomized guinea pigs may have potential for some bone density reduction but studies in other species report that ovary removal does not have significant effects on the serum levels of calcium regulating hormones, parathyroid hormone and the vitamin D metabolites nor do they indicate changes in physical activity, or increases in bone fractures over the longest protocols to be used in these studies (up to 160 days). Careful observation of the general health and activity of the animals will assist in detecting unlikely, but possible, long term bone density-related ailments.

Animals will be monitored daily for signs of ill health such as weight loss, becoming pale, breathlessness, lethargy and fur standing up in a reflex response that can signal ill-health. The food and water intake will be checked by a trained, experienced technician.

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

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

For animals experiencing each protocol the expected severity is moderate.

What will happen to the animals at the end of the study?

- Killed



Replacement

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

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

There is no alternative to the use of animals for these investigations. We are investigating complex inter-relationships between various physiological systems i.e. blood pressure regulation, blood pumping by the heart and nerve and hormonal signalling which all contribute to the pathophysiology of heart failure. These inter-relationships would be too complex and ill-defined to be studied using computer modelling or cell culture models.

What was your strategy for searching for non-animal alternatives?

Long-term cultures of animal adult heart muscle cells and use of human heart cells were considered for this work.

Why were they not suitable?

Long-term cultures of heart muscle cells are not suitable for the *ex vivo* / *in vitro* studies because they cannot divide to form new cells. Adult heart muscle cells cannot be cultured for longer than 48 hrs because thereafter they revert to a fetal (ie not adult) form and lose the mature systems that couple the electrical excitation of the cell to the mechanical contractile response. These are the systems we are studying. The electrical and mechanical changes to large and failing hearts can only be carried out on cells that have undergone, or are undergoing, the cellular processes involved in these transitions.

Human cardiac cells can be obtained from valve replacement or heart transplantation surgery and can be used for these studies. However they are difficult to obtain consistently and the patient history and past treatments can produce many confounding variables. Acquiring control tissue is fraught with obvious ethical and resource issues.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 is calculated using published information, previous experience (taking into account experimental difficulty and success rates) and the biological variation encountered in making some of the common measurements (e.g. electrical and contractile parameters). Our calculations account for different time points of study and give us assurance that our experimental methods will detect an effect (assuming one exists).

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?



We follow the international PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines, have taken advice from our statisticians and have planned the studies to enable them to be published according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines which are intended to improve the reporting of research using animals and approved by the National Centre for the Replacement, Refinement and Reduction of Animals in Research also known as NC3Rs.

What other measures apart from good experimental design will you use to minimise numbers?

We are fully aware of the cost/benefit issues this work entails and constantly strive to refine techniques and maximise the quality of the experimental data. We minimise live animal use by capturing as much *ex vivo* and *in vitro* data as possible from each animal. Most of the experimental work is carried out *ex vivo*. The use of single cells and multi-cellular preparations allows different experiments to be carried out on one heart by the sharing of the heart tissue digest with different scientists so minimising animal use. In addition, *in vivo* echocardiography enables characterisation of the structural and functional changes occurring to the heart as a consequence of hypertrophy and heart failure in the conscious, rather than the anaesthetised, animal avoiding re-use and multiple doses of anaesthesia and so reduces animal numbers.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We need to induce heart failure so that we can study the cellular processes that underlie the condition for a more tailored and effective approach to treatment strategies. Appropriate choice of animal model is crucial for meaningful scientific outcomes. We use an animal model that closely resembles the human situation. We produce heart enlargement and heart failure in the guinea pig by performing constriction of the main artery leaving the heart. This procedure replicates the extra load on the heart that it experiences in overcoming high blood pressure (common in humans), is well-described for the guinea pig and is well-established in our laboratory. The surgery is under aseptic conditions and incorporates effective anaesthesia, analgesia, and fluid replacement to minimise pain and suffering. End points are chosen so that distress is reduced to the minimum consistent with the symptoms of onset of the condition.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Adult guinea-pig is the least sentient species that importantly shares the physiological features that are under examination with human.



We are investigating complex inter-relationships between various physiological systems i.e. blood pressure, heart output and nerve and hormonal signalling which all contribute to the pathophysiology of heart failure and to sex differences in onset of the failure. These inter-relationships would be too complex and ill-defined to be studied using computer modelling or cell culture models or in immature animals.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We have noted and incorporated a number of refinements to each protocol since we started to use the guinea pig 20 years ago. We continue to monitor animals closely, and with the Veterinary Surgeon constantly assess possible improvements in post-operative care, pain management, and animal environments.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We follow international PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines, plan and conduct studies according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and use National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs) guidelines to ensure our animal experiments are as robust and reproducible as possible.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We read the monthly updates from the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs) on their events and publications and their e-Learning resources all of which detail advances in replacement, refinement and reduction techniques and best practice and provide information how to put these in place.

Explain the choice of species and the related life stages

It is vital to choose an animal model that closely resembles the human situation. The guinea-pig is the least sentient species with which we could carry out these experiments and have confidence of achieving meaningful data. Among the small mammals commonly used in these types of study, the adult guinea-pig is the species of choice because in many relevant physiological aspects it displays similar features to the human. Our interventions in this animal produce well-defined changes to the lungs and chambers of the heart that mimic the clinical condition closely. The electrical and mechanical processes of individual heart muscle cells are almost identical to that of human. In terms of sex hormone balance and production, again the guinea-pig is similar to the human. Finally, we have long-standing experience with the cardiac physiology of this species providing us with a solid empirical reference point for our control groups.





75. Investigating the neural circuits of hearing-in-noise

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

The aim of this project is to better understand the neural circuit(s) that underlies hearing-in-noise. In addition, we aim to understand how hearing-loss and aging affect the function of this circuit(s).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 ability to hear-in-noise substantially declines with hearing loss and as we age, this makes it difficult for sufferers to engage with the world and can leave them socially isolated and lead to a decline in mental health. This work is important as it seeks to enhance our understanding of the neural circuit(s) of hearing-in-noise (a critical skill for communicating as most environments contain noise). In addition, we aim to better understand how neural hearing circuits adapt with age and hearing loss. By understanding how these circuits change we may be able to provide critical insights into how we can maintain normal function in these circuits, allowing people to have normal hearing for longer.

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



We will advance our understanding of the neural mechanism(s) of hearing, specifically hearing-in-noise and how these pathways are affected by age. These results will be disseminated through conference proceedings and peer-reviewed published articles. The data described in these publications will also be made publicly available for other researchers to use.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Short term: We will use these results ourselves in our studies of hearing loss to inform subsequent work. In addition, these data will be directly shared with collaborators to improve models of auditory function.

Medium term: We anticipate that others will also use these results for a variety of purposes related to studies of basic auditory processing and efforts to model and simulate the function of the auditory system.

Long term: These results could form the early foundation of the detection of specific sub-types of hearing loss, e.g. dysfunction of descending auditory feedback, and potentially lead to research targeting treatments to reduce the impact of hearing loss.

How will you maximise the outputs of your work?

We will seek at every stage to collaborate with computational modellers, this will allow experimentation in silico, substantially increasing the range of hypotheses that can be tested. Work will be disseminated at conference both by ourselves but also through our collaborators. Where possible we will aim to pre- register publications allowing us to publish the work even when unsuccessful, reducing the amount of duplication in the field.

Species and numbers of animals expected to be used

- Mice: 300

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, mice will have an intracranial injection of viral vector (AB, recovery surgery, ~47% of animals) and then 4-6 weeks later undergo physiological measurement under terminal anaesthesia (AC) and perfusion. A subpopulation (~36%) of animals will also have hearing loss induced (AB) followed by a wait of typically 4 weeks and then have their hearing function measured (AB), then another wait of at least 3 weeks before having the viral vector injected (AB, recovery surgery with a 4-8 week wait) and then physiological measurement under terminal anaesthesia (AC) and perfusion. The remaining animals (~17%) will undergo physiological measurement under terminal anaesthesia (AC) and perfusion.



Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Intracranial injection: May rarely lead to infections in animals recovered from this procedure. This will be treated with antibiotics or if necessary the animal schedule 1 euthanised. The duration of effects should be brief (<2 weeks).

Stitches or bonding glue may become loose: If this occurs the wound will either be fixed immediately or the, if not possible, the animal euthanised. If the wound is resealed and comes loose a second time the animal will be euthanised.

Recovery surgery: May cause post-operative pain and discomfort. The first 3 days after surgery will be treated with analgesic. If signs of pain appear after suspension of this treatment postoperative analgesic will be continued (through consultation with the NVS).

Induction of hearing loss: Noise exposure may, very rarely, cause ear discomfort from inflammatory reactions to noise-induced cochlear damage. We will administer post-procedure analgesic but if this problem persists the animal will be euthanised.

Ageing mice up to 15 months: Mice may attack one another when co-housed for long periods. If this is detected the aggressor may be single housed, if this single housing produces stereotypic behaviour it may be necessary to euthanise the animal.

Animals expressing fluorescent proteins: Cre recombinase or opsins will be used. The animals with fluorescent proteins or CRE inserted in their genome proposed here do not usually express any obvious detrimental phenotype and have no apparent disabilities. However, unexplained deaths do occur in both wild type and GA litters.

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

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

Mouse = Mild (17%), Moderate (83%)

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

The aim of this project is to understand the function of specific neural pathways formed by auditory circuits. To understand how these pathways, respond to sounds we need to be able to record from them while sound is being presented. This requires a normally functioning ear and brain as well as a way to monitor neural function at the cellular level.



At present the only way to do all of these things is through in vivo physiology. In addition, to establish the causal role of these pathways we need to be able to functionally manipulate them, this can only be done (in a specific manner) using transgenic techniques. Taken together these two points mean that animal use is unavoidable.

What was your strategy for searching for non-animal alternatives?

Sensory neuroscience research generally relies on four main approaches to answer questions: 1) non- invasive behavioural experiments, 2) computational modelling experiments, 3) in vitro physiology 4) in vivo physiology. All of these approaches were considered as potential tools for addressing the aims of this work.

Why were they not suitable?

Non-invasive techniques work on a gross population level and do not offer cellular and circuit (i.e. within a cortical column) level measurement of function. In addition, they do not allow specific functional control of parts of the neural circuit and so cannot answer fine grained circuit level questions. Computational techniques can work at the cellular and circuit level and allow modelled functional control. However, to make these models more than conceptual (i.e. biologically plausible) parameters within the model need to be constrained by biology. At present there are large and fundamental gaps in our knowledge about projections from auditory cortex including: the basic parameters needed to model their intracellular dynamics, how they respond to sound, how they connect (motifs) to their targets, how they modulate function in their targets and how they change with age. The only way to gain this knowledge is to study these projections in real brains. In vitro physiology allows study of actual brain function at the cellular and circuit level and also allows functional control of real neural populations. However, this work aims to study the involvement of the neural circuit in listening behaviour. This means animal use is unavoidable because brain slices lack the real sensory inputs from the outside world and connections from other parts of the brain.

However, within these limitations, both computational and in vitro methodologies will be used wherever possible to inform the in vivo studies.

Reduction

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

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

In order to ensure reasonable estimates of the required animal numbers we used data from existing research papers as a guide to the potential size of changes to neural firing rate that we might expect to observe. We also used our previous experience of these types of experiments to help us estimate the number of neurons we might reasonably expect to measure in each animal. Taken together this was sufficient to create an estimate of the total number of animals required for each experimental condition and controls, to ensure the experiments were designed to be able to deliver statistically robust results. It should be noted that all animal numbers reflect the maximum number of animals that might be needed. The results of objective 1 and 2 could dramatically reduce the number of



animals needed. For example, the results of objective 1 could remove the need to study brainstem projections (therefore a maximum of 4 pathways would be investigated in objective 2), likewise, objective 2 could further reduce the number of pathways under investigation (i.e. if the critical pathway has already been found).

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Experimental design: We will re-derive power calculations after the first few experimental animals, this will help improve estimates and probably reduce the number of animals we will eventually use. We will work through the pathways systematically; this means if a particular pathway is yielding results this will become the primary region of focus for these studies. We feel this approach could dramatically reduce the number of animals investigated. We will consult widely within the department, with the NC3Rs Regional Programme Manager and use NC3Rs resources (e.g. I currently use instructional videos like "How to pick a mouse" to help train staff) within the NC3Rs resource hub to ensure good experimental practice is used. Finally, when selecting the projections to target we will work methodically from most to least probably involved in hearing-in-noise.

Animals: Where possible we will use animals from one experimental group as sham surgery controls for other experimental groups. Projection neurons can be broken down into subtypes, we will use "catch all" transgenic lines and use strategically placed injections to gain specific control of subpopulations. This approach means that multiple subtypes can be accessed in the same animal, which in turn means fewer animals will be used.

Brain imaging: When possible we will employ brain imaging prior to recordings. By imaging prior to recordings we will be able to map out the known subdivisions of auditory cortex. This means more accurate positioning of recording electrodes increasing the data yield, improving the precision of the data and reducing the number of animals used.

What other measures apart from good experimental design will you use to minimise numbers?

Pilot studies will be employed to test the feasibility of or improve the quality and efficiency of future studies. Where possible tissue will be archived for potential later use (particularly in older 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Viral vector injection: At present the most humane way to target specific sub-types of cells (e.g. specific subtypes of projection or inhibitory neurons) is to use genetic approaches combined with viral vector injections. In some cases, e.g. with projection neurons, it is



possible to use wild-type mice to specifically label projection neurons and so this approach could be used if transgenic lines were not possible. However, this approach requires two invasive surgeries (two sets of intracranial injection) rather than the one employed here and hence represents more pain, suffering and distress to the animal. To avoid this transgenic REDACTED will be employed.

Induction of hearing loss: Animals will be anaesthetised during hearing loss induction and monitored closely after surgery for any signs of discomfort (which will be treated with analgesics if found). The only other way to produce noise induced hearing loss would be in conscious animals, therefore, this approach is the most refined.

Measurement of hearing function: Hearing function could also be assessed using chronically implanted electrodes, however, this approach would require much more handling of the animal and an invasive surgery (moderate procedure). For the approach used here the animal will be anaesthetised during the procedure, will not need to be regularly handled and it is a mild procedure.

Mouse model: We will use transgenic REDACTED that either originate from or are backcrossed with C57BL6 REDACTED to help ensure no harmful phenotypes are encountered. In addition, this mouse model is known to develop premature hearing loss. In fact, hearing loss generally associated with old age can be observed in healthy adult mice. By using this model we do not need to age mice to the point where they develop noticeable health problems.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice represent the only animal model with "normal" mammalian hearing and that allow this level of specificity of control of cortical circuits. Alternate transgenic models exist, for example drosophila melanogaster, however these animals do not have mammalian style hearing organs (i.e. a cochlea) and so it is difficult to translate findings in this animal model to human hearing. In addition, in vitro work does not allow the inclusion of a functional ear (critical to the type of work proposed here).

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Protocols have been written to allow, time permitting, either measurement or induction of hearing loss under the same anaesthetic. This will reduce the number of anaesthetics needed for each animal and reduce overall suffering.

Animals will be monitored for several days after surgery and induction of hearing loss to increase the speed at which any potential problems may be discovered. In addition, daily weight records will be made for several days following these procedures. This will increase the probability of picking up problems quickly, allow careful monitoring of the recovery trajectory (animals will be "tagged" during surgeries to allow individualised care for group housed animals) and reduce the amount of suffering experienced by each animal.

Long gaps in time (minimum of two weeks but typically longer) will be left between procedures to allow for full recovery and reduce the possibility for interaction between anaesthetic events or procedures.



What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will use LASA “Good practice” guidelines throughout. In addition, we will also follow ARRIVE guidelines when designing and carrying out the studies and publishing work.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will stay abreast of developments and advances in 3Rs guidelines by visiting the NC3Rs website periodically. I am subscribed to the NC3Rs newsletter and have (and will continue to) engage with the NC3Rs Regional Programme Manager to develop ever improving research methods.

Explain the choice of species and the related life stages

Our hearing ability changes during our lifetime, therefore, we will use mice with normal hearing, i.e. in the age range of juvenile to early adulthood (p10-50) and also mice with hearing loss, i.e. middle/old age (p51-540). This range of ages is necessary to monitor the development of auditory cortical circuits and capture how their function changes throughout the lifetime (and allow sufficient time to allow hearing loss). This will allow us to understand the critical developmental steps that underlie hearing-in- noise and also the critical function that is lost as we age.

Transgenic mice are critical to this work as they offer the control of highly defined cell types, when combined with viral vector injections, within specific brain regions. This is critical to allowing specific subtypes of auditory cortical projection neurons to be targeted.





76. Investigating the pharmacology of advanced drug delivery technologies and conventional medicines

Project duration

5 years 0 months

Project purpose

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

Key words

advanced formulation strategies, nanotechnology, long-acting

Animal types	Life stages
Mice	adult
Rats	adult
Rabbits	adult
Hamsters	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 is the aim of this project?

We aim to improve the effectiveness of medicines by developing better ways of delivering the drugs to the sites in the body where they are needed to work. Drugs investigated will include currently used drugs and new drug candidates with potential application in Covid-19 and other diseases. New drug formulations, (the way in which drugs are combined with other substances) and drug delivery devices will be created. We will use animals to measure the concentrations of drug in the blood and other tissues following administration of the drug, and we will be able to predict which drugs would give us the best results in humans. The data using animals will be used to inform computer models which will inform future human drug trials.

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



Why is it important to undertake this work?

The work undertaken in this project will improve our understanding of what factors in the body can influence how an administered drug works. An exciting area of research in this project will be the continued investigation of the benefits of using advanced formulation strategies (e.g. nanotechnology) to improve the effectiveness of drugs treating human diseases. Novel formulations can improve the ability of the drug to enter the body and to allow for long-acting treatments. This reduces the need for patients to take multiple pills and can also lower the cost of treatment. It is hoped that our research completed during the last 5 years will be the foundation for this project to continue this important field of research and continue to prove the application of novel formulations in humans, including in repurposing existing medicines for Covid-19 treatment and prevention.

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

At the end of this project we would expect a number of peer-reviewed publications in high impact journals that guide clinical practice in the treatment of diseases important to public health, chronic diseases and resistant organisms.

At the end of this project we would like to see a change to currently available products for the treatment of diseases such as SARS-CoV2, malaria, TB, HCV and HIV. These products should be made available in low and middle income countries as well as high income countries to ensure those with the highest need have access to safe and effective treatments.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The work undertaken in this project will improve our understanding of what factors in the body can influence how an administered drug works. If we understand the factors influencing how a drug works in the body, we can work with chemists to design drugs that work more effectively, or to improve current drugs using advanced drug delivery systems. These changes to how drugs work in the body can improve a patient's experience of taking their medication, potentially by decreasing side effects, changing how often they have to take the drug, etc.

Peer reviewed publications will be produced throughout the duration of the project to ensure that the research we carry out informs the scientific and clinical community as soon as possible, this ensures the data is relevant and any changes made are based on up to date data analysis.

Any products generated as an outcome to this project will take longer to make an impact as they will have to undergo rigorous regulatory testing and clinical trials before they would be available products on the market. However, the data from this project and early phase clinical trials will be published.

How will you maximise the outputs of your work?

We aim to generate publications for high impact journals to ensure the data are widely distributed throughout the scientific community. We will also generate publications to demonstrate unsuccessful approaches, allowing the scientific community to use our data to inform their future experiments or techniques.



As a group we lead a consortium of scientists investigating long-acting medicines for the treatment of malaria, TB and HCV and we have been working on improving HIV treatments for over 20 years. Recently, as an antiviral group, we have used our resources to help the global effort to find a therapy for COVID-19, working closely with other scientists locally as well as throughout the UK and the world.

Our group has close links with the wider scientific community, including funders, regulators and community advocacy groups to help achieve the aims of the project as well as disseminate the information generated via international scientific meetings and community action groups.

Our group will expand over the coming years to allow for the recruitment of PhD students and scientists to use the data generated from this project to inform future scientific directions, enabling collaboration and further knowledge into the future.

Species and numbers of animals expected to be used

- Mice: 1300
- Rats: 1200
- Rabbits: 430
- Hamsters: 700

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

For the majority of animals used for this project an experiment will last between 1 and 90 days depending on the nature of the regimen to be tested.

For medicines which will be assessed for their distribution after a single dose animals will usually be in an experiment for 24 hours. Animals will be administered a medicine either by an injection (into the tail vein or flank for example) or by administration using oral gavage and placed back in the home cage. At specific time points after administration

Rodents may be placed under anaesthesia for blood to be withdrawn via cardiac puncture and then administered an overdose of anaesthetic agent (usually the case for mice).

Rodents may be placed in a heatbox to dilate veins and then placed in a restrainer for blood to be withdrawn from the lateral tail vein before being placed back in the home cage.

Rabbits would be placed on a heated table and ears would be rubbed gently to encourage blood flow, a butterfly needle would be inserted into the ear vein to withdraw blood.

Animals will have blood withdrawn a number of times in 24 hours but the total volume withdrawn is a maximum 10% of circulating blood volume in any 24 hour period.



Animals will be anaesthetised usually using isoflurane prior to an overdose of an injectable anaesthetic.

For long-acting medicines assessment will follow the same pattern as above except time points for blood withdrawal will be spread across up to 90 days, ensuring the total volume withdrawn is a maximum 10% of circulating blood volume in any 24 hour period and 15% in any 28 day period. Long-acting medicines are typically administered via injection into the leg muscle, by microarray patch or by implant and in most cases this will be completed under inhalational anaesthesia.

Experiments to investigate medicine distribution in the bile and other sanctuary sites will involve surgery and animals will be administered a medicine, usually orally, and will be placed under anaesthesia for the entire duration of surgery. Animals will not be allowed to recover from surgery and will be killed using an overdose of anaesthetic so as to minimise any pain, suffering or distress.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Animals may experience some discomfort, abnormal behaviours, significant weight loss or other indicators of poor health, but these will not prevent normal feeding and drinking or other normal activities other than for short periods. These experiences are not expected in any animals and work carried out prior to animal work will inform the experiments as much as possible to minimise the discomfort experienced by the animals.

The most likely expected adverse effect on animals in this project is pain during a procedure. For example, pain during insertion of a needle when administering a medicine intravenously or when inserting a needle for blood withdrawal. This pain will be very short lived and transient and will not affect the animal's overall wellbeing. Any administration of a substance that is thought may cause longer lasting pain, for example intramuscular injection, will be completed under anaesthesia and pain relief will be administered at the same time.

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

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

Protocol 1 - non-recovery - 100% of mice and rats used will experience this severity

Protocol 2 - Moderate - 60% of mice, rats and rabbits used in this protocol are likely to experience a moderate severity

Protocol 3 - Moderate - 75% of mice, rats and rabbits used in this protocol are likely to experience a moderate severity

Protocol 4 - Mild - 100% of mice, rats and rabbits would experience this severity

What will happen to the animals at the end of the study?

- Killed



Replacement

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

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

Animals are required to achieve the aims of this protocol because they have genetic and biological characteristics closely related to humans, making them a good model for the evaluation of drug characteristics. They allow us to simulate tissue sub-compartments of relevance for human disease, especially sanctuary sites such as the gut, central nervous system and rectal associated lymphoid tissue.

What was your strategy for searching for non-animal alternatives?

We use a combination of in vitro, ex vivo and in silico techniques to investigate the pharmacokinetics and distribution of medicines and novel formulations of active pharmaceutical ingredients (APIs). We also use *Galleria mellonella* to investigate long-acting drug release and depot architecture. We are also developing an in vitro muscle model to investigate the pharmacokinetics of medicines which show long-acting capabilities in cell culture experiments.

Why were they not suitable?

These models are not suitable as they have the following limitations:

Inability to simulate tissue sub-compartments of relevance for human disease (especially sanctuary sites such as the gut and rectal associated lymphoid tissue and central nervous system)

Inability to faithfully simulate human biology and the processes that are involved in the absorption, distribution, metabolism and excretion of API's

Immune cells examined ex vivo may not faithfully simulate the situation in vivo

Available models may not include all the cell types or immune molecules that may have an important impact on the medicine exposure or response relationship

Galleria mellonella are proving difficult to extract medicine from to measure drug concentrations and as they do not have the same level of genetic similarities with humans as animals, these would only be a preliminary screening tool

An in vitro muscle simulation is in its early phases of development and at the moment cannot be used to investigate long-acting injectables

Reduction

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



numbers consistent with scientific objectives, if any. These 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 tend to use groups of 3-4 mice to obtain an estimate of central tendency (usually the mean) and variance. These estimates are adequate for the downstream analysis to be completed. We know on average how many experiments we are likely to complete per year based on our previous work. We have also taken into account the current pandemic as this will likely increase our animal numbers as experiments are being completed at a much faster rate than usual to respond to the global need.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We have an in depth understanding of the variance structure around much of our data, which has helped us design our studies. We use the following general principles:

We attempt to define exposure-response relationships using in vitro models to reduce the numbers of animals required to establish these relationships.

Simulations of pharmacokinetics are employed when appropriate to inform in vivo experiments in order to minimise the number of animals used and to maximise the data generated from each experiment.

We have altered the length of studies if later time points do not provide any additional information. This reduces the number of animals that are required.

What other measures apart from good experimental design will you use to minimise numbers?

We debate study design as a collective to ensure we arrive at the best possible design.

We seek statistical help and critique for our model 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Rats and mice will be used. Rabbits will be used for validation purposes only. Some will only experience being administered a general anaesthetic, after which the whole experiment will be completed under anaesthetic and the animals will be killed without recovering consciousness. Other animals will receive doses of the potential new medicines being tested after which blood and urine samples will be taken at intervals to assess how the animals re-distribute the medicine within their bodies and how long it takes for it to be



eliminated eg in urine. Body tissues will be collected at the end of the experiment after the animals have been humanely killed. The medicines to be given are not expected to cause the animals suffering and the sampling methods do not involve more than transient discomfort. Some animals will be anaesthetised in order to give the medicine by certain routes eg into the muscle. At the end of the experiment some animals will be anaesthetised so that a larger volume of blood may be collected and then the animals will be killed without recovering consciousness. No animal is expected to experience more than what is regarded as 'moderate' adverse effects and most will experience only mild and transient discomfort.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

In cases where it is appropriate and in some protocols within this project animals will be used that are terminally anaesthetised so as to minimise any discomfort. Mice will be used rather than rats where appropriate and rabbits will only be used for validation purposes. Mice are the lowest sentient animal we can use in order to mimic the human response.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals will be monitored very closely after administration of a drug to ensure no acute reactions take place.

Animals will be kept in the procedure room for monitoring at this early stage to ensure an anaesthetic machine is available if needed to minimise any suffering that may occur.

Animals will be checked regularly throughout the day of the experiment by both the REDACTED and PILs and animals will be humanely killed if deemed necessary.

Animals are kept in a post-operative room after any surgical procedure and monitored by a PIL.

Animals will be administered pain relief if needed and will be administered pain relief prior to any surgical procedure.

Animals will be transferred in the enrichment tube when possible to decrease distress.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

NC3Rs website

Diehl et al, J Appl Toxicol, 2001, 21(10):15-23

Laboratory Animal Science Association (LASA) Guidelines

Understanding Animal Research website

Recent publications in our field of study

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We regularly attend NC3Rs conferences and meetings held at the establishment and nationally to update our knowledge and ensure techniques used are in the best interests of



the animals. Any information from these meetings is disseminated to the group at weekly group meetings and changes will be put in place, led by myself or my 'deputy'.

Any PIL leading a study will check the NC3Rs website, as well as other relevant literature, to ensure that the procedures necessary for the study are carried out using techniques that are in the best interests of the animal's welfare. PILs will also seek advice from the REDACTED team, including the NACWO and NVS.

Explain the choice of species and the related life stages

Rodents such as mice, rats and hamsters have genetic and biological characteristics closely related to humans, making them a good model for the evaluation of drug characteristics. Rodents allow us to simulate tissue sub-compartments of relevance for human disease, especially sanctuary sites such as the gut, central nervous system and associated lymphoid tissue as described in this protocol.

Rabbits are important as a model of validation in a second species. Rabbits are an important species for prodrug validation as rabbits are a more representative model of hydrolase activity compared to a mouse or rat. Hamsters are especially important for investigation into Sars_CoV2 virus as they have been found to be a very good model of the disease.

All rodents will be used at a weight of between 20 - 25g which correlates as between 5-7 weeks old, therefore, they are at the adult stage. Hamsters will be used at 80-120g (approximately 5-8 weeks of age). Rabbits will be used at 3kg (approximately 14-18 weeks of age), this ensures that growth of each animal has stabilised.





77. Investigating the Regulation of Glucose Transport

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The single most important action of insulin is to promote glucose transport into skeletal and cardiac muscle and fat. This is achieved by the regulated trafficking of glucose transporters from intracellular storage compartments to the cell surface. We seek to define the mechanism(s) responsible for this trafficking.

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

Why is it important to undertake this work?

Insulin-stimulated glucose transport is impaired in people with Type-2 diabetes. There are > 420 million people living with diabetes. Diabetes is a major cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation, is the 7th leading cause of death world-wide and costs the UK £10billion/year. Defective glucose transport lies at the heart of this disease and our work seeks to define the impairment in mechanistic terms.



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

Understanding how glucose transport is regulated in physiologically relevant cell systems is key to the development of novel therapeutics and/or identification of new potential drug targets to treat diabetes, ameliorate its consequences or assist in patient stratification.

Our work will:

Identify novel potential mechanisms for the enhancement of our basic understanding of insulin action

Offer new targets for therapy,

Potentially help with stratifying patients with diabetes [Type 2 diabetes is a heterogeneous disease, and improving classification is considered essential if precision medicine is to be developed for this condition].

We will seek to publish our findings in peer-reviewed open-access journals and to disseminate our work via STEM-events, peer-peer networking, Twitter and Diabetes-UK-led outreach events.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

This work may have potential benefits to people living with diabetes, as it may identify new therapeutic targets or help stratify the disease. However, this is basic science and as such a translational end point is likely to be many years in the future.

How will you maximise the outputs of your work?

The applicant has participated widely in outreach and public-facing activities to publicise his work. These include REDACTED-led events, Diabetes UK led events such as talks with patient support groups, and he has also participated in the REDACTED International Science Festival (twice).

We collaborate widely within the Glucose Transport field, participate in regular meetings and actively seek to publish our work in a range of journals which reflect the 'highly visible' output of exciting new findings, to the 'solid' output of important but arguably less novel data that nonetheless provide the field with important insight and comparative data.

Species and numbers of animals expected to be used

Mice: 300

Predicted harms

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



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

All work proposed here will use transgenic mice. The typical GA mouse in question expresses a fluorescent protein tagged glucose transport in muscle and heart and exhibits no adverse effects as a consequence.

Groups of 10 mice will be placed on high-fat diet or 10 on a normal diet for up to 20 weeks (but some may be used from week 12 depending on body weight gain and blood glucose levels) as only around 2 mice can be used per week because of limitations on cell processing discussed above.

Blood samples will be collected to confirm development of insulin resistance; a difference between control and high-fat fed animals of >0.7 mmoles/l will be used as a cut off, or an increase in body weight of greater than or equal to 20%.

At no longer than 20 weeks after the start of the experiment, animals will be humanely killed using a Schedule 1 or a non-Schedule 1 method.

Up to 50 mice in total will be on a high fat diet and a corresponding number will be used as control mice.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The reporter gene the mice have does not cause them any health problems and mice don't exhibit any adverse phenotype. Breeding animals are kept for a maximum of 12 months of age.

Cardiomyocyte isolation and intact muscle dissection will be performed once the animal has been humanely killed.

Some animals will be placed on a high-fat in order to induce insulin resistance, for a maximum of 20 weeks. Mice on this modified diet will be inspected daily and are likely to become obese, they will also be monitored for signs of ill-health, including changes in respiration, dehydration, activity, unkempt appearance, abnormal posture, and trembling.

Repeated micro blood sampling will be carried out whilst animals are on the dietary modification experiment, and this is classed as Mild.

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

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

Breeding of colony – Sub Threshold.

High-fat fed induction of insulin resistance - Moderate.

Blood sampling classed as Mild.



What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

Insulin action, and defects associated with diabetes and obesity, are multi-system diseases. This intra-organ communication cannot be replicated in any cell culture-based model.

The analysis of the plasma membrane dispersal of glucose transporters using digital stochastic optical reconstruction microscopy (dSTORM) requires the use of fluorescently tagged transporters. To achieve this in primary cardiomyocyte or skeletal muscle culture systems requires post-isolation infection with a virus to drive expression of the transgene. In our hands, the time taken to drive the transgene is too long for the cells to maintain insulin responsiveness and they de-differentiate. Hence, the use of a transgenic mouse strain expressing the tagged transporters obviates this step and represents the only viable experimental system available to us.

Moreover, the ability to modify the insulin resistance status/diabetic status of the animal is not possible in any cell-culture based system.

What was your strategy for searching for non-animal alternatives?

Preliminary work has employed cultured cell lines, which have paved the way for this work.

Why were they not suitable?

None of the cell culture lines come close to recapitulation of cardiomyocytes isolated freshly from intact hearts. Our attempts to use primary cardiomyocytes from other species have failed as a consequence of the need to over-express a gene which cannot be achieved in cardiomyocyte cultures in vivo as the cells do not survive. The transgenic line we propose to use was developed to circumvent this issue.

Reduction

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

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

All work proposed here will use a specific transgenic mouse unless other similar GA mice offering a better model become commercially available. The mouse in question expresses



a fluorescent protein tagged glucose transport in muscle and heart and exhibits no adverse effects as a consequence. Details of the strain are at: <https://www.jax.org/strain/027496> A breeding colony will be established; the animals are homozygous so genotyping is not required and there will be fewer waste animals.

Much of the work proposed will involve the development or refinement of imaging approaches which depend on the reagents used (e.g. in tissue digestion for optimal cardiomyocyte isolation, different batches of enzymes need to be evaluated before experimental work can begin) and experimental set-up on the microscopes.

Our estimation of numbers is based on a planned use of 1 mouse per week in the development phase (months 0-24), and then groups of mice for specific experimental hypothesis testing, once optional conditions for analysis are established.

1 mouse/week months 1-24; it is anticipated these will be from the maintenance breeding colony. The colony will be maintained at approximately 3 pairs with 4 breeding stock held. Breeding will be staggered to reduce overproduction and wastage.

If conditions from the development studies indicate that methodology and analysis can be achieved, then we will also examine the impact of diet-induced obesity/insulin resistance. Groups of 10 mice placed on high-fat diet or 10 on a normal diet for 20 weeks.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

One animal per week in the initial period of study will allow us to develop/apply methods developed using cultured cells and will supply sufficient material for considerable experimental analysis.

Cardiomyocytes will be isolated using standard procedures and intact muscles will be isolated from the same animal post-mortem. Thus each animal will contribute tissues to both plasma membrane dynamics analysis and whole tissue transporter Mesoscale morphology analysis, reducing the numbers required for the project.

What other measures apart from good experimental design will you use to minimise numbers?

One animal per week in the initial period of study will allow us to develop/apply methods developed using cultured cells and will supply sufficient material for considerable experimental analysis.

Cardiomyocytes will be isolated using standard procedures and intact muscles will be isolated from the same animal post-mortem. Thus each animal will contribute tissues to both plasma membrane dynamics analysis and whole tissue transporter Meso-scale morphology analysis, reducing the numbers required for the project.

Refinement

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



Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

All work proposed here will use a transgenic mouse. The mouse in question expresses a fluorescent protein tagged glucose transport in muscle and heart and exhibits no adverse effects as a consequence. Details of the strain are at: <https://www.jax.org/strain/027496> A breeding colony will be established.

Most procedures will be performed under terminal anaesthesia without recovery.

We will induce insulin resistance by feeding a high fat diet to mice, this is a well established way to create a diabetic mouse.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The specific use of this particular transgenic mouse is required. No other model is available which can answer the scientific questions of this project as well as this one.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

This strain of GM mice is bred specifically for analysis of transporter biology in muscle cells. It imposes no harm or disability to the animal which are by all measures assessed normal and healthy with no ill effects.

Animals fed a high-fat diet in order to induce diabetes will only be kept on the modified diet for a limited period of time (max 20 weeks) and thus are not expected to develop significant adverse effects. They will be given plentiful access to water and their bedding will be changed frequently so as to keep them dry and comfortable. Their glucose levels and general condition will be closely monitored and if any develop defined adverse clinical signs, they will be humanely killed.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Local guidelines for blood sampling will be used and the NC3Rs website will be regularly consulted.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Training courses are provided regularly by local staff and external colleagues. I will keep up to date with more refinements via the NC3Rs website.

Explain the choice of species and the related life stages

Transgenic mice, as outlined above, offer the only viable route for these kinds of experiments. The starting age of 5 weeks of age is the recommended one for this model.





78. Investigation of epigenetic reprogramming in male germ cell development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

The overall aim of this project is to identify factors (genetic or non genetic) that direct epigenetic reprogramming (epigenetics refers to non-genetic influences on gene expression), and development of the male germline, during sperm development and early life.

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

Why is it important to undertake this work?

Germ cell development, for the most part, unfolds during embryonic life, when germ cells are present in small numbers and are extremely difficult to access for manipulation. The consequences of any defect during this process has long-lasting and destructive consequences, reducing one's ability to produce offspring and/or resulting in birth defects in future offspring. Although our study primarily focuses on the basic biology of germ cell development, a better understanding of the processes could have an impact on both reproductive medicine and treatment of rare non-genetic hereditary diseases. While it does not pose any immediate health risk, infertility has a deep impact on the life of an individual,



his/her family and, more broadly, society. Worldwide, 15% of couples of reproductive age (15-44 yrs) have experienced problems conceiving a baby. The cause will be different (genetic defects, surgical removal of the testis/ovaries, sterility inducing treatments e.g. chemotherapy, unknown factors), but they often result in either a failure to produce functional gametes (sperm/eggs) and / or difficulties in carrying a pregnancy to full term. To date, approaches for the in vitro differentiation of gametes from patient derived stem cells have not been fully successful, mostly due to large grey areas in our knowledge of the events directing this key developmental process. At the same time, the underlying cause of several hereditary diseases still remains unknown in most cases, due to an inability to identify the causative genetic mutation(s). The absence of a known cause has therefore greatly impaired the development of treatments, which often have been targeted to alleviate the symptoms rather than reversing the primary issue. Understanding how non-genetic hereditary diseases are passed to offspring could provide a new framework in which to identify and investigate the causes, and the origins of these.

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

Completion of this project will offer new insights on the dynamics, mechanisms and relevance of epigenetic reprogramming in directing germ cell development. Although all the cells of an organisms share the same genetic information encoded in genomic DNA, different cell types are defined by the expression of different sets of genes. This is controlled by a second layer of information called epi- genetics ("epi" is a Greek prefix for "on top of"), whose functional elements are chemical modifications of the DNA bases or the histones (the proteins that "wrap" the DNA. It is key for the proper development of an embryo with a full compendium of tissues and cell types, that the content of the epigenetic landscape from both the egg and the embryo is reset to a blank state to avoid any developmental bias. In particular, we will focus a large part of our efforts and resources to obtain a better understanding of the events leading to the specification of Spermatogonial Stem Cells (SSCs), the sole contributors of gametes in the adult testis. Since SSCs are ideally positioned to be relay points of hereditary information transmitted to the next generation, data on the mechanisms guiding this fate decision may offer novel insights into how and when epigenetic traits can be transgenerationally inherited in mammals.

We foresee publications in peer reviewed journals to arise from the project proposed here.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short term, the impact of these outputs will mostly be seen in fuelling basic research on the mechanisms of germ cell development and the mechanisms by which non-genetic traits can be inherited through generations. Such increase in knowledge may lead to uncovering the causes and mechanisms of rare diseases, possibly guiding the development of new therapeutics.

By identifying the events that guide immature germ cells to commit to Spermatogonial Stem Cells, in the long-term this work may provide the theoretical basis for the development/refinement of therapies aimed at treating male infertility by promoting the production of functional sperm using drugs or other treatments. This project could also provide unique insights on the local niche of developing germ cells, it is plausible to expect a similar application of the scientific outputs to generate healthy sperm from patient derived stem cells in a petri dish.



How will you maximise the outputs of your work?

We will be open to collaborate with other laboratories that have interesting models, data or questions where our methods can be applied. Moreover, all the knowledge and expertise generated with this project will be spread through talks, conferences and publications.

Species and numbers of animals expected to be used

- Mice: 11800

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Throughout this project we will produce mice, often with a genetic modification. In most cases we expect the genetic modification to cause only male sterility (possibly associated with a reduced female fertility). Any other effects of the genetic modification for new lines of mice may be unknown (except in the case of reporter mice where there would be only labelling of a gene, and no additional effects would be expected). We do not expect the modifications to be harmful for the mouse. In some cases we will produce embryos (instead of live full term animals) by allowing the mice to become pregnant. A mouse may experience injections of chemical agents at locations appropriate to each agent. This may typically require 2- 3 times in the lifetime of an animal. The mouse will then be killed later on in the time course of the experiment.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Our intention is to carry out this project without adverse effects on the mice, however in a small number of cases mice may experience weight loss or show signs of abnormal behaviour. This should last for no more than a week.

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

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

The majority will be mild, as they will only experience the breeding protocol. Of those being used on an experimental protocol (approximately 3% of the total animal proposed), < 5% may experience a moderate severity if they suffer adverse side effects to any agent we deliver.

What will happen to the animals at the end of the study?

- 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?

Germline development is a highly integrated process involving constant cross interaction between germ cells and their surrounding cells (within the gonads). Development from a germ cell to a fully functional sperm cell proceeds via a series of signals from both the germline and also surrounding cells, part of which are still left uncharacterised. For these reasons, it is necessary study germ cell development in an in vivo setting.

What was your strategy for searching for non-animal alternatives?

Previous work of our laboratory attempted to establish a protocol to reproduce male germ cell development in vitro with the goal of driving mouse embryonic stem cells to acquire the identity of mature germ cells, also referred therein as in vitro differentiation. In such protocol, we would solicit the generation of germ cells either via series of chemical induction events alone or in combination with co- cultures of in vitro differentiated germ cells and primary non-germ supporting cells sourced from mouse testis.

Why were they not suitable?

Unfortunately, our efforts proved unsuccessful because we were unable to obtain fully and robustly differentiated germ cells. After careful consideration, we attributed our failure to two main factors. First, in vitro differentiation protocols mostly rely on strong and acute inductive signals that fail to recapitulate the highly integrated and dynamic developmental process that cooperatively define a true germ cell as it appears in vivo, and for some of which we still don't have a comprehensive understanding. Second, in vitro differentiation protocols are extremely sensitive to experimental variability (e.g. different batches of reagents, experimental conditions and timings), whereas living organisms have evolved strategies to actively ensure robust and reliable physiological processes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 experiment in each of our objectives, we have carried out a statistical sample size calculation which is detailed in the project plan. In general, we have based our estimate of the size and variance of the data that we expect to acquire on similar experiments performed by our laboratory in the past. Where this has not been possible we have clearly specified so in our project plan, and we will perform a pilot study to better characterize the data which will inform a proper statistical calculation of the sample size.

We have also consulted in house statisticians for guidance.



What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We have attempted to follow the best available guidelines on experimental design, including these listed on the NC3R website. In addition, we screened the existing literature to inform ourselves on the best standards currently applied in terms of sample size determination.

Wherever possible, we have attempted to perform multiple measurements from the same dataset (applying the appropriate corrections) and to compare multiple treatments to the same control, reducing the number of control animals required.

We based our sample size calculations on estimates of effect size and variability from previous data of our laboratory. However, this is just an initial estimate, and we fully expect that our understanding of the data will increase over the project. As this happens, we will refine our sample size calculations and either use less animals, if possible without reducing scientific significance, or seek permission to enrol more animals in the study if we realize that a higher number of replicates is needed.

What other measures apart from good experimental design will you use to minimise numbers?

We aim to use efficient breeding strategies and seek to use tissue from each animal for as many different data analyses as possible.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use laboratory mice of several different strains, some of which are genetically modified in order to either i) tag certain cells of the body with a fluorescent molecule or protein tag or ii) to induce sterility, in order to study the development of germ cells. None of these modifications will cause harm to the animals. In order to study the behaviour of germ cells within their biological context in the gonads, we will use chemical agents to trace them throughout development or to specifically label molecules within these cells at specific times. These agents are frequently used and well tolerated and we expect very few animals to display unwanted side effects.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice are the least sentient, and most understood (in terms of gene modifying and cell labelling) animals that represent mammalian development. Some of our data will be collected from adult mice as we require full biological development of the animal.



What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We will use several strategies, refined over the course of our previous work, to minimize suffering for the animals. While producing transgenic animals, we will use methods and breeding schemes that maximize the number of animals with the correct hereditary traits that are produced, this will ensure that as few as possible transgenic animals are born unnecessarily. The facility creating the transgenic mice now use a more refined methodology with far improved success rates over those used previously. When administering substances to neonates, we will leverage on the expertise of other users in the institute how they have refined the route, volumes and protocols for dosing.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow the guidelines listed within the public resources available from the NC3Rs website. We will follow all of these, as well as several other standard operating procedures that were developed by a team of specialists at our institute for the explicit purpose of minimizing animal suffering, and are periodically updated. While designing experiments, we will follow a series of guidelines existing in the literature to design and report our experiments, in addition to the ARRIVE and PREPARE guidelines from the NC3Rs, and will consult with an on-site statistician to ensure that we're using as few animals as possible for our study.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Our institute routinely circulate advances in the 3Rs and we will always seek to identify ways these can be incorporated in our project, while ensuring they do not effect the (statistical/ biologically relevant) consistency of our data collection.

Explain the choice of species and the related life stages

We are using mouse models to understand the mechanisms that direct the correct development of germ cells in the testis to produce healthy and fertile sperm. Previous work from our laboratory and others has shown that the mouse male germline closely recapitulates the same key events and processes that drive human germ cell development in men, with very few exceptions that will not interfere with our proposed work. Since most of the epigenetic reprogramming of the germline, the focus of our project, unfolds during embryonic and early postnatal life, our study will require the use of embryos, pregnant females and neonates in addition to adult individuals that will represent the end time point of the process of our interest.





79. Investigation of the factors involved in the development and maintenance of myeloid malignancies, and ways to target them therapeutically

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

In this project, we want to understand why specific blood cancers start and develop. These cancers, called myeloid malignancies are more frequent in older persons, but the reasons for that are unknown. Our goal is to understand why the elderly develop myeloid malignancies more frequently, how to identify people at a particularly high risk of going on to a more severe condition and how to prevent or block the disease from progressing.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 blood cancers called myeloid malignancies (MM) are increasingly common with age. With a frequency of more than 15 cases per 100,000 people per year, they represent an important health issue and with the ageing of population worldwide, the number of patients will largely increase. The majority of patients still die from their disease as treatments have not improved over the years. The main obstacle to improving the treatments is a lack of understanding of how these diseases begin and progress to more severe forms. There is therefore an urgent need to develop new therapies, and this is one of the main goals of our project.

Due to the current lack of effective treatments of myeloid malignancies, another approach is to prevent them. Prevention was not an option until recently for MM as the patients often get ill suddenly, without any earlier signs of the disease. However, recent work by our team and others has found that we can identify people at high risk of developing these cancers many years in advance. Therefore, an early detection may be possible which opens a field to a search for preventative methods. Prevention of myeloid malignancies is another main goal of this project.

Myeloid malignancies originate from haematopoietic stem cells (HSCs), the cells responsible for blood cell production. Under daily pressures, HSCs acquire mutations in their genetic material which changes the information contained within it. Whilst the vast majority of such mutations do not affect HSC behaviour, certain mutations can equip an HSC with advantages. In consequence, this HSC will increase production of its daughter blood cells out of proportion, a situation known as clonal haematopoiesis (CH). Clonal haematopoiesis is driven by a group of mutations associated with Myeloid Malignancies (MMM). Importantly, CH becomes increasingly common with age and is detected in more than 30% of people aged 70 years and older. Most people with CH do not develop myeloid malignancies, however, they are at an increased risk.

Apart from mutations changing the behavior of HSCs, it is clear that other factors influence their behaviour. There is now increasing evidence that the pace at which an HSC and its daughter cells grow, is affected by ageing and factors such as infection, inflammation, diet, exercise, sleep and the microorganisms co-living inside the human bodies. The impact of ageing is particularly strong for some of the mutations, as the blood cells with these mutations are only able to over-grow out of proportion in old but not in young people.

It is not known how the ageing and the other factors affect blood cells with a mutation. In this project we aim to find which early events enable HSCs with different MMM to begin its uneven growth and progression to malignancy. Preventing and blocking these processes are the main objectives of our work.

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

This project will provide new knowledge about the factors involved in the initiation and progression of a group of blood cancers called myeloid malignancies. This new knowledge will enable to develop new laboratory and bioinformatic tools to test people at increased risk of myeloid malignancy and predict their disease progression. Furthermore, we aim to find new or existing drugs to prevent and treat myeloid malignancies (cancers), in order to improve patient survival and quality of life.

The new knowledge gained in this project will be shared with the scientific community through conferences and publications. We will also remain involved in public engagement activities to increase awareness about our research to the wider public.



What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short-term, the medical, scientific and pharmaceutical communities will benefit from our research by using our findings to develop new strategies to identify, prevent and treat myeloid malignancies.

To the rapid benefit of patients, we plan to work along with other scientists, medical doctors and biomedical companies to speed up the application of our findings into new testing methods and new treatments. We have a good experience in such processes and we are already advanced with trials of drugs discovered through similar studies.

In the longer term, we hope that our work will lead to the development of new methods for the prevention and treatment of myeloid malignancies and related blood cancers. Prolonged survival and a better life quality of patients with myeloid malignancies and their families will be the most direct and measurable benefits from our research. In the broad sense, ensuring a better treatment options will have a positive effect on society at many levels.

Our groups have a longstanding interest in public engagement activities and we will remain actively involved in these to promote the understanding of our research in line with the programme of public engagement proposed by our institution.

How will you maximise the outputs of your work?

Our findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings. The generated data will be archived and made openly available to other researchers around the world via the European Nucleotide Archive (ENA) at EMBL-EBI. Our new animal models as well as viable cells collected from the mice will be valuable to other scientists studying the myeloid malignancies. We will distribute our mice and other tools freely to non-commercial establishments after publication. Pre-publication access will be extended to other scientists as part of collaborative studies. We have already exported a number of our mouse models to several leading universities and institutes worldwide. We will also aim at share and use tissues from aged mice using SHARM (<https://www.sharmuk.org>).

Species and numbers of animals expected to be used

- Mice: 33100

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Most methods and procedures required for this project are well established in the field of blood cancer research and in our group; we have successfully used them in the past to characterise different mouse models of leukemia.



In practice, mice will be bred to generate offspring with changes of interest in their genetic material, so-called genetic alterations. Frequently, to achieve an active state of the genetic alteration, a mouse will need a specific induction procedure involving a single or repeated injection of a substance that will turn on or off the genetic alteration. Some of these experimental mice, after activation of genetic alteration, may undergo ageing up to 125 weeks. Throughout this time we will perform blood collection from mice on one or multiple occasions to assess how the number of blood cells change as one of markers for the disease progression in response to various treatments.

Frequently, the treatments will aim to mimic the exposures normally encountered by humans during their lifetime such as low-grade inflammation following infection or poor choice of diet. The treatments may last up to 12 weeks and are expected to produce long-lasting but virtually undetectable changes, in the same way that occurs in humans. To this end, mice may undergo a pro-inflammatory or anti-inflammatory treatment with different substances to modify the behaviour of the changed blood cells or their normal body environment. The treatment may involve a single or multiple administrations of a substance (e.g. bacterial cell elements) by different delivery routes (e.g. in the food, injected under the skin). The frequency of these treatments and volumes used are not expected to harm the animals in the long term. Similarly, mice may undergo a procedure of behavioural modification to indirectly influence the activity of blood cells. To this end, mice may undergo a change in their dark-light cycle, receive a modified diet or may be provided with exercising equipment.

Some mice will undergo irradiation to compromise or remove their own blood system before a transplantation of blood cells from another mouse. Transplantation will be performed only once in the life time of a mouse. Often, preconditioning (e.g. irradiation) of an animal may be necessary to facilitate growth of transplanted cells. Depending on the amount of irradiation, mice may experience post-irradiation illness but our advanced care plan will effectively limit this condition. Where relevant and available, human blood cells may be transplanted into mice to confirm the findings from studies in mice. Frequently, the transplanted cells may be pre-manipulated "in a dish" prior to transplantation to modify their potential to generate leukemia in mice.

On occasions, the pre-manipulation on cells "in a dish" may enable emission of a bioluminescent light by the cells to enable imaging of the cells growth within the body. Upon injection of an imaging substance in the mice, this light can be captured from the living mice placed in the imaging system. To enable stress-free restraint of the mice for imaging, they will undergo a short term anaesthesia for a brief period and are expected to recover from anaesthesia without longterm harm.

Anti-leukemic treatments may be administered to the mice to prevent, slow down or eliminate leukemic cells. Examples of leukemia-targeting substances include drugs that have been shown to effectively inhibit tumour cell growth when tested "in a dish". Frequently, the treatments may involve repetitive handling of mice for a brief moment to administer the substance by injection. However, the frequency of these treatments and volumes used are not expected to harm the animals in the long term.

On occasions, a large volume of blood may be required for analysis and to obtain this, a mouse may be induced under terminal anaesthesia (a state of deep sleep terminated by euthanasia) to enable a collection of a terminal blood sample. Similarly, terminal anaesthesia may be used in an animal to perfuse it (supply through circulation) with fixative solution for preservation of its organs for further analyses.



Mice will be killed by one of the approved humane killing methods. Upon killing of a mouse, we will collect different organs (e.g., bone marrow, spleen, liver) to analyse multiple parameters such as proportions of different cell types, gene expression, protein expression etc.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

General health monitoring is performed for all mice by trained personnel in the animal facility and members of our research team. According to our long-standing experience with our mouse models of blood cancers, we understand in-depth the possible adverse effects of our experimental designs and can enhance animal care for the mice at increased risk of ill-health. Any mice showing signs of ill-health or noticeably reduced activity for more than 24 hours will be humanely killed. In addition, when visible signs of compromised wellbeing are observed, enhanced monitoring and support will be put in place according to the care plans included in this license.

During our project we will use different procedures and experimental setups which are expected to have a various level of impact on animal wellbeing as listed below.

Breeding of genetically altered mice is not expected to cause harmful effects. Nearly all of the genetic changes used for our project will be seen in the adult animals used for our experiments. Therefore, the majority of the animals used for breeding will not experience adverse effects which might arise from changes in their blood system.

Induction treatments are meant to activate genetic alterations. Activating substances will be injected in mice up to 7 times every day or every other day. A mild weight loss up to 10% of pre-treatment weight may appear in relation to biological effects of injected substances. These adverse effects will decrease or stop once the induction period is finished and will not affect the mice long-term.

Blood collection will be used to obtain samples for tracking of a disease progression and other analyses. This will be done up to 20 times during a life time of a mouse with at least 2 weeks between the bleedings. The bleeding may lead to a short-term discomfort and stress but is not expected to affect the animal in long-term.

Blood cancer will develop in some of the mice. Over the weeks or months, these mice usually show gradual changes in the number of blood cells, which may cause an increase in the size of the spleen and occasionally, in liver size. Infrequently, other tumours may appear. Their growth will be judged by external signs such as swelling of the tummy or appearance of masses under the skin. Mice may appear hunched (a specific posture with a hump in the back), their hair may stand on end and they may show reduced activity at the advanced stages of the disease. These signs will be humane endpoints.

Transplantation (a transfer) of blood cells will be done into some of the mice. This is a standard method to assess the potential of cells under challenging conditions. Tail vein will be used most frequently to inject the transferred cells. A transient discomfort and stress due to temporarily holding an animal, as required for injection, may lead to a short-term discomfort and stress behaviour. However, the procedure itself is not expected to negatively affect the animals in the long term.



The injection of abnormal blood cells into the cavity in a long bone (thigh bone) may be used in specific cases to improve the outcome of the transplantation or modify the experimental outcome. This is a surgery and is discussed below under a paragraph 7 about surgeries.

Preconditioning (preparation) may be needed for some mice before they get a transplantation. Irradiation will be used most frequently but other methods may be used as well. Irradiation may induce a post-irradiation illness and a level of the illness depends on the amount of irradiation (a dose) received by a mouse. The doses will be always adjusted to ensure the least dose is used therefore limiting animal suffering.

Low dose of irradiation may cause a mild drop in weight of mice (up to 10% of pre-irradiation weight). The reason for this is a decreased interest in food and water. The activity of mice should remain unchanged. Irradiated animals will be checked daily and weighed at least twice weekly to monitor their health. An enhanced pre- and post-irradiation care plan will be provided if necessary. Our experience and the published data show that the mice recover fully from the irradiation-related adverse effects within 7-10 days. The mice should return to their weights within 7-10 days post-irradiation.

High dose of irradiation leads to a long-term failure of blood system and therefore, the irradiation illness is more pronounced. Mice may drop in weights up to 15% of their pre-irradiation weight.

Moreover, mice may be less active. The mice should return to their weights and activity within 7-10 days post-irradiation.

Sometimes, only a part of a mouse body will be irradiated. These mice will be put under 20-30 minutes of anaesthesia (a state of a deep sleep). This short anaesthesia will not result in long-term adverse effects.

Preconditioning will be done only once in a lifetime of a mouse.

Surgeries will be done in germ-free conditions to prevent infection and under anaesthesia (a state of a deep sleep). Following surgery, mice will be kept warm until they are fully recovered and moving freely around the cage. The mice are expected to recover from anaesthesia within 20-30 minutes at most and no long-term effects are expected. Medication to relieve any pain will be administered to the mice while they are anaesthetised for the surgical procedures. Mice should be fully active within 2 hours from surgery. Surgeries should not result in long-term adverse effects. Difficulties to regain activity beyond 2 hours post-anaesthesia or signs of more than mild pain and distress will be humane endpoints.

Imaging may be performed on some mice to visualise a cancer growth. Injection of a substance for imaging will be done before each session. Each session will last up to 45 minutes. At most, we will do 9 sessions three times a week. Imaging will be done under anaesthesia (a state of deep sleep) and the mice are expected to be fully active within 20-30 minutes post-anaesthesia. Imaging is not expected to result in long-term adverse effects.

Different anti- or pro-disease treatments will be used to change the progression of the blood cancers under study. We will use only substances with known properties and known to cause up to moderate adverse effects such as reduced activity, decreased feeding and



weight loss up to 15% of pre-treatment weight. These adverse effects may persist throughout the whole period of treatment. The mice may receive the substances in drinking water, food or by injections.

Physiological anti- or pro-disease treatments will be used to mimic states normally met during a human lifetime. Examples of such treatments include a high-fat or a nutrient-deficient diet, a free exercise, inflammation, infections, changes in intestinal bacteria and disruption of the light-dark cycle. These treatments may result in mild disturbances to the normal condition of an animal such as mild changes in their activity and weight gain or loss up to 10% of their pre-treatment weight. These treatments will last up to 12 weeks.

Ageing as a physiological process is not expected to have more than mild effects on mice. General physiological features of ageing such as reduced activity, a weight change, hair loss or blindness may appear and persist until the animal is killed. The ageing mice will benefit from an enhanced care plan and killed immediately if they experience any signs of suffering. Rarely, different cancers may develop in the oldest animals and an existing blood disease may progress faster in aged mice.

Unless otherwise specified, the work in this licence will be undertaken in accordance with the principles set out in the Guidelines for the welfare and use of animals in cancer research. British Journal of Cancer (2010) 102, 1555-77.

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

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

Below is the summary of animal numbers used on different severity protocols:

Moderate severity, 12100 mice:
protocol 3: Embryo recipients 1000 - all mice will reach moderate severity

protocol 4: Vasectomy 100 - all mice will reach moderate severity

protocol 6: 4000 moderate, 75% predicted to reach moderate severity (3000)

protocol 7: 3000 moderate, 75% predicted to reach moderate severity (2250)

protocol 9: 4000 moderate, 75% predicted to reach moderate severity (3000)

Mild severity, 21000 mice:

protocol 1: superovulation 2000 - all mice will reach mild severity

protocol 2: generation of founders 1000 - all mice will reach mild severity

protocol 5: breeding 15000 (this number includes animals that will be moved to other protocols) protocol 8: 3000 mild, 75% predicted to reach mild severity (2250)



What will happen to the animals at the end of the study?

- 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 are particularly interested in the biology of blood cells carrying different mutations (i.e. changes in the genetic machinery that controls the cell) causing a specific type of blood cancers called myeloid malignancies. In myeloid malignancies, blood cells with mutations escape the normal regulation of blood system and out-grow other types of blood cells leading to an unbalanced blood production. According to studies from ourselves and others, this process of unbalanced growth is strongly linked to the ageing but it is not known how.

In many ways, human and murine ageing are physiologically similar, which allows to use results from research on human and mouse interchangeably and validate the research relevant for human in mouse models.

Regulation of blood system is not fully understood. It is of particular interest, but highly challenging, to address the regulatory environment controlling the blood stem cells (i.e. the cells that will produce all subsequent cells) and all the produced downstream types of blood cells. Multiple approaches have been proposed to re-create "in a dish" the interactions between blood cells and their regulatory factors. However, these attempts have not been fully successful and only a limited number of known regulatory clues can be combined. Only a living organism provides a complete structure required to study biology of normal and diseased blood system.

Due to the technological progress, mice can be used to generate models of mutations found in patients with blood cancers. Blood system of mice has been thoroughly studied and therefore, it offers a well- validated resource to research on development and function of normal and mutated blood cells. Of note, differences between normal and diseased cells are frequently subtle and animal studies may provide a more restrictive test condition.

Studies in mice have a strong track record in facilitating basic and translational (patient-oriented) cancer research in ways that cannot be pursued in "in a dish". Our work moves increasingly in a therapeutic (treatment based) direction and it is essential for us to test potential treatment approaches in a living animal. This step is necessary before we are confident that the tested therapy is appropriate for humans.

What was your strategy for searching for non-animal alternatives?

In our project, we combine different approaches to answer different questions. The majority of our work is performed "in a dish" where we grow immortalised cells (cells that do not die) as established cell lines. Equally, we use in cultures a material obtained directly from mice e.g, different types of blood and blood-supporting cells.



Human material from healthy people and from patients plays essential role in our project and we have in place arrangements to obtain fresh and archived samples. Human blood and blood-supporting cells will be used for cultures and for different direct analyses.

Newly generated data as well as existing datasets are analysed using bioinformatics (the use of computers to analyse and interpret animal and human data). This helps us to solve our research questions as well as to propose new directions.

Why were they not suitable?

Due to the limited knowledge about regulation of blood system within both mice and humans, it is not possible to re-create the physiological conditions to culture blood cells "in a dish". Once removed from the body, blood cells rapidly lose their properties and their biology cannot be fully studied "in a dish". The culture systems are a simplified and unphysiological context, where only specific questions are studied without the full picture. By contrast, studies in living animals allow scientists to draw physiologically relevant conclusions. Findings from cultured cells always need to be tested in living animals to validate their relevance.

Bioinformatics analysis of our own and publicly available data is extremely important for our research and three members of our team work full time as bioinformaticians. Their work helps us to direct our research towards specific questions and goals, but we will always need to validate their conclusions in cellular systems, living animals and 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?

Keeping the numbers of animals to a minimum is an important goal that we constantly strive to achieve. This begins during the planning of an experiment. We use results from previous experiments and statistical calculations to accurately estimate the maximum numbers of animals required to answer our different experimental questions. We understand that underestimating animal numbers can be just as wasteful as overestimating them.

In our project, almost half of all the animals will be used for breeding to generate animals for experiments. For some of our research questions, we need 3-4 different genetic alterations to be present within one animal. Obtaining such mice often requires a number of breeding steps and therefore, we expect a large number of animals to be used for breedings. We will always aim to keep the breeding schemes as efficient as possible.

Regarding the numbers of experimental animals, we base our estimates on past studies within our groups. We will calculate the numbers of the mice required for different types of experimental designs using statistical tools appropriate for the purpose and with an adequate power. For example, in experiments designed to test therapeutic effect of an anti-leukemic drug, we will estimate a number of animals using calculators designed



specifically to match the data output required. In experiments where we compare changes with a normal distribution, we will use calculators using t-test statistics.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

In general, we will consider the PREPARE guidelines to formulate a study and to control the quality of each of its parts. According to a good laboratory practice, we will write a protocol for each experiment including a description of the experiment and number of animals per group. Researchers will be advised to design their experiments using the NC3Rs Experimental Design Assistant (EDA). Online tools, for example easy-to-use spreadsheets, may be used as a guidance to estimate the number of animals for particular types of experiments (<https://www.bu.edu/researchsupport/compliance/animal-care/working-with-animals/research/sample-size-calculations-iacuc/spreadsheet>) or <http://www.biomath.info/power>, recommended by the 3Rs-Reduction.co.uk website <http://www.3rs-reduction.co.uk>). In case of doubts, we will search an advice from an expert statistician.

Experimental plans will be clearly communicated to the animal facility staff to ensure a straightforward operation. Experiments will be conducted to enable publication of results in open access journals and in accordance with the ARRIVE guidelines. We will consider publication of negative data to avoid unnecessary duplication of the work by others. Sequencing or genotyping data will be archived at EMBL-EBI's European Nucleotide Archive (ENA) which is openly accessible to any researcher around the world.

We have and will keep updated with methodological advances; reduce the numbers of animals needed to design statistically sound experiments, as for example using single cell technologies or bioinformatic analysis tools as they are developed. To reduce the numbers of animals needed, we will also use cells from the same animal to answer different experimental questions.

Where there is any doubt, advice from a professional statistician will be sought.

What other measures apart from good experimental design will you use to minimise numbers?

Wherever possible, we shall import existing mice strains with altered genes of interest or obtain animals from other project licences with authority to transfer animals rather than generating new ones, as this will reduce significantly the numbers of mice used in the generation of new genetically altered mice. However, if there is strong justification for different variant of a gene or a different genetic background, we will generate the new model using the most efficient technology.

We frequently require mice with a combination of two up to four genetic modification. Generation of a mouse with such complex genetic image (genotype) requires a high number of mouse pups to be born. We adapt mating strategies to maximise the numbers of mice born with the required genotypes. We have adapted our mouse mating strategies to maximise the numbers of mice born with the required genotypes, and whenever possible, we will use mice homozygous (i.e. have two identical genes) for mutation if these animals are fit and fertile.

We will interact closely with the staff of animal facility to ensure that the breeding of our genetically altered REDACTED is as efficient as possible. We will carry out regular reviews



to check that only required REDACTED are kept alive. If there are no plans to use a mouse line for at least a year to come, the line will be cryopreserved (reproductive material collected from mice will be deep frozen for storage). To improve recovery rates from frozen material, cryoprotective additives will be used during freezing according to the current best practice. Historically the cryopreservation of sperm from some mouse strains, has been highly problematic due to post-thawing recovery rates. Additional media constituents allow us to use sperm as a suitable archiving option. By utilising this option we can again minimise the numbers of females used at this stage of the process.

Furthermore, cryopreservation will allow saving REDACTED in case of a large-scale problem affecting the animal facility, e.g. disease outbreak.

Our REDACTED will be available for sharing with the wider scientific community. This will help reduce the number of animals used to generate lines by other scientists.

To reduce the number of animals required to detect a true biological effect, we will aim to use litter-mates without genetic alteration as controls. Such control litter-mates coming from the same parental pair have their genetic material most similar to test animals and are exposed to the same environment. Their usage counterbalances the problem of variability between mice from different litters, and therefore, it decreases the overall number of animals needed to reach valid results.

Whenever possible, we will maximise the amount of data obtained from the same experimental animal by collecting different types of samples and information (e.g., organ size, blood counts, tissue). This will enable combining of different types of results on a per mouse basis, helping to reduce variability.

When planning new types of experiments, we will search through literature if any data on similar approaches is available and we will contact other researchers for expert advice.

Ageing of experimental mice is essential for our project and we will aim to efficiently use the aged animals. We will collect different types of samples from aged mice and we will share unused tissues through ShARM UK.

We will equally publish negative data to inform the scientific community about inefficacy of tested treatments to avoid others doubling the animal 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Throughout this project we will use mice with different mutations (i.e. changes in their genetic material) to study why specific blood cancers start and develop. These blood cancers are called myeloid malignancies as they result in cancerous overgrowth of myeloid blood cells.



There are over 30 mutations linked with myeloid malignancies and our groups have previously generated and studied a number of mouse models carrying these mutations. Over the years, we have developed extensive expertise in these models and in the types of studies which we intend to carry out. Therefore, we understand how to obtain valid experimental outcomes in mice with progressing blood cancers without causing unnecessary suffering to animals.

In most of these models the mutation is not active and the animal will not experience any harm. Once the mutation is turned on by an appropriate treatment, the mouse is expected to gradually develop signs of abnormalities in the blood system. As the timing of mutation activation is under our control, we are able to date when the disease should begin. This way, we can intervene early to minimise adverse effects. Most of mutations that we study will lead to diseases with a slow progression which again, enables us to keep progression of adverse effects to minimum.

In this project, we will continue to study our mouse models in a new perspective of ageing. To this end, we may use mice with genetic alterations resulting in ageing features. For example, mice with genetic alterations in immune or nervous systems or with changes in metabolic functions may be used. Whenever introducing a new model to our studies, we will collect all the information needed to assess any special needs of the new model. According to this information and if needed, we will design an appropriate care plan.

Lastly, most of our studies are based on material collected from animals post-mortem and therefore, will not involve animal suffering.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Blood cancers that we study affect mostly aged people. However, according to data from us and other researchers, mutations responsible for these cancers may appear in middle-aged people many years before the patients start to show the disease. Furthermore, only a small part of people with mutations will develop the cancer. In consequence, our research questions are, firstly, why only some people with mutations go on to the cancer and secondly, how advanced age promotes the disease. Furthermore, blood cancers of our interest have slow progression and therefore, passage of time is essential to model their development. Mice, due to their similarity to human physiology and their life time to around 2 years, allow to address our questions in a physiologically complete system within a feasible time frame.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

In this project, we will perform different types of experimental procedures. The highly trained staff at the animal facility offers significant assistance and ideas for refining protocols.

In general, post-procedure checks are put in place to monitor the condition of animals in the hours after they have undergone a procedure. For each procedure, we will put in place an appropriate care programme to minimise harms to the animals as described below.

Surgery



Surgeries, for example intra-bone injections (directly into a bone), will be done under general anaesthesia (a state of deep sleep). After surgery, mice will be kept warm until they return to their normal activity. Mice will receive medication to relieve any post-surgery pain.

Ageing

Wellbeing of ageing animals will be carefully monitored by regular checks of their condition. The standard check will include observation of their activity, skin and fur condition, teeth, posture and weight. The observations will be crosschecked with the previous report to detect any sign of deterioration. When deterioration in the condition is observed, appropriate countermeasures will be taken and may include: supportive diet, additional cage enrichment or specific treatments (e.g. medicaments applied on skin) upon consultation with animal care specialists.

Housing

Mice will be maintained in individually ventilated cages (IVC) where clean filtered air is provided in a controlled way to reduce anxiety in mice. To satisfy natural social instinct of mice, they will be kept in groups. Group sizes will follow the regulations to avoid the cage overcrowding. Separation and housing of single mice will be introduced only when aggressive behaviours are found.

Each mouse will be marked by its earmark and will be tracked in the computer system to enable easy access to all the information about the mouse.

Enrichment

Mice will be kept in deep bedding and will be provided with nesting materials and 'play tunnels'. Particularly sensitive mice will receive an additional cage enrichment to reduce their stress levels.

Genotyping

Genotyping is performed to confirm presence of genetic alterations in mice. As material for genotyping, we will use ear-clips taken for identification of mice.

Cancer models

In most of the cases, genetic alterations causing the cancer will be activated in mice only upon a specific treatment. As the timing of the alteration activation is under our control, we are able to predict when the disease should begin and intervene early to minimise adverse effects.

Treatments to mimic ageing-related conditions

We will mimic ageing-related conditions by multiple approaches including induction of mild inflammation, infection, disturbed dark-light cycle or changes in diet. These treatments may lead to weight drop as an adverse effect. The weights of mice will be checked at least twice weekly. To counteract the weight drop, mice will receive enriched diet before and throughout the treatment period to keep their feeding.

Irradiation



Irradiation is a standard method to prepare mice before transplantation. Following irradiation, the mice suffer from post-irradiation illness where decreased food and water intake are main adverse effects and may lead to weight drop. With higher doses of irradiation, the symptoms may be more pronounced. Mice will be weighed before irradiation and every other day to detect weight drop as a sign of ill-health. Weighing of mice will continue until the weights are stabilised. To prevent the post-irradiation illness and weight drop, mice will always receive enriched diet at least two days before irradiation and during the post-irradiation period until their weight is stabilised.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Webpage <https://www.nc3rs.org.uk> is our reference for the best practice in animal research. Various aspects of work with animals such as handling, anaesthesia, analgesia, blood sampling, euthanasia and welfare assessment are covered there under the link:

<https://www.nc3rs.org.uk/topic-specific-resources-0>

Equally, we will refer to <http://www.lasa.co.uk/> where under the tab: current publications we find "Guiding principles" for different aspects of working with animals. Particularly rich information can be found there regarding the principles for aseptic surgery:

http://lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf

To generate numbers of animals matching our experimental needs, we will follow the assessment framework available from Home Office under the link:

https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773553/GAA_Framework_Oct_18.pdf

For the best practice, all experiments will be planned in agreement with PREPARE guidelines (<https://norecopa.no/prepare>) and we will follow ARRIVE guidelines (<https://norecopa.no/3r-guide/arrive-guidelines>) to report our animal research.

All PIL holders working under this PPL will be strongly encouraged to research through and implement these resources in their experiments.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will stay informed about advances in the 3Rs by following information in the newsletters prepared periodically by our establishment Named Information Officer. I also have an account at <https://nc3rs.org.uk/> to receive updates from NC3R and equally I follow information published in the Lasa forum https://www.lasa.co.uk/current_publications/ and use the Establishment's 3Rs Search Tool.

All team members will be encouraged to become acquainted with these resources. Additionally, any information relevant to the welfare of the mice under our care will be forwarded to all group members during our lab meetings and as brief handouts. We will always aim to set a good example to our colleagues. We will work closely with staff in animal facility to ensure the best care for our animals.

Explain the choice of species and the related life stages



Mice are widely used for biomedical research due to their anatomical, physiological and genetic similarity to humans. Furthermore, they are easy to maintain in laboratory environment. The enormous progress of technology over recent years enabled us to efficiently generate mouse models useful to study myeloid malignancies which are our main research interest. Our mouse models carry changes in their genetic material (mutations) copying or resembling the changes in genetic material of patients with myeloid malignancies.

Myeloid malignancies are a group of blood cancers affecting mostly aged people. Consequently, effects of the ageing on the blood system are essential part of our project. In many aspects, the ageing of mice resembles the ageing of humans and therefore, mice are extremely useful resource to understand the human ageing. The life-length of a mouse averages to 125 weeks which allows to complete the project goals within a reasonable time-frame. Equally, we will use young adult mice as a reference for results obtained with aged mice.

Our work is focused on blood system and will benefit from a well-established mouse-specific range of methods. For example, blood cells can be easily analysed and separated by flow cytometry, a method where a set of the cell molecules is specifically marked to emit light under certain conditions and subsequently used to describe the cell for further analysis.





80. Investigative & Enabling Safety Assessment Studies

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

No answer provided

Animal types	Life stages
Rats	juvenile, adult
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 is the aim of this project?

The overall aim of this project is to generate data to support the development of potential new medicines, with the following key elements:

Characterisation of functional and toxicology liabilities related to a specific agent/compound, a pharmacological target/class or a chemical series to allow selection of optimal candidate drugs for further progression, refine drug discovery strategies and provide appropriate animal models to enable effective candidate drug selection.

Investigate drug development strategies or mitigate observed unexpected toxicities. This may include, for example, investigating novel dosing strategies to improve agent/compound tolerability and effectiveness or combination strategies where potential new medicines may be dosed alongside existing therapies.

To provide support to development of new medicines and improved welfare through Method Development or Enabling work in the following ways:

Method development to enable an investigation.

Method development to increase the predictability of in vivo studies.



Method development to validate new/existing techniques in literature or in other laboratories.

Bespoke studies to assess potential refinements for animals e.g. evaluating specific enrichment methods

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

Why is it important to undertake this work?

Studies performed under this project licence will contribute invaluable data to support and progress the development of potential new medicines where there is a clinical unmet need. These studies will be fundamental to the elucidation of a safety risk associated with a target/agent early in its development, and therefore support decision making with respect to the progression of a candidate molecule towards the clinic.

Drug development is a long and costly process. It is therefore desirable to avoid unnecessary expenditure of time, money and resources as well as any unsubstantiated use of animals on substances that are not suitable for development as potential medicines. This can be achieved by testing agents/targets early in the drug discovery process, leading to early removal of unsuitable agents/targets from the pipeline or making a scientific assessment of an agent/target safety risk, alongside a strategy to mitigate observed toxicities whilst still delivering benefit to patients.

Furthermore, it would be unethical to administer novel substances to humans before making every effort to ensure that they were safe. In all disease therapy areas, developing novel agents with any associated toxicity into a new medicine can ultimately limit the benefit to the patient. This is particularly relevant to cancer patients where such toxicities may be dose limiting, i.e. they cannot tolerate higher doses of agents to improve efficacy because of adverse effects.

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

Data generated from studies performed in support of discovery and early candidate selection will be used internally to allow decision making on whether to develop drug candidates and, with other preliminary studies may help to define doses for subsequent studies. Data from studies will enable the investigation of safety liabilities identified through *in silico* (computer program simulated tests) and/or *in vitro* (in cells) methods and will be pivotal to the progression of novel anti-cancer agents and other therapeutics. Data from experimental studies will also be used to develop ways to mitigate, monitor or manage unwanted effects in the clinic, for example, by changing dosing regimen, previously intolerable adverse effects can be reduced, allowing a patient to continue with a beneficial treatment. Some studies will typically be performed to provide data in support of dose selection, typically tolerability and/or toxicokinetics (levels of drug in the blood or tissue), or to build/validate a new or different methodology thereby enabling other parts of the safety evaluation programme to proceed with best possible outcomes.



For all of the above, data will feed back into programmes for future compounds to refine discovery and development strategy and thus avoid unnecessary animal use and expenditure.

For compounds which progress, these studies may be included in regulatory submissions as supporting studies.

Data from studies may be included in peer review publications or presented at internal and external meetings and conferences.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Early indications of safety and toxicity liabilities, including functional endpoints where appropriate, will allow early detection of unsuitable molecules and thus support decision making with respect to the progression of the a novel molecule, ultimately supporting the development of only the best new medicines towards the clinic for the benefit of patients where there is a clinical unmet need.

How will you maximise the outputs of your work?

Data from studies performed under this licence will feed back into research programmes for future compounds to refine discovery and development strategy and thus avoid unnecessary animal use and expenditure.

For compounds which progress, these studies may be included in regulatory submissions as supporting studies where they have informed for example a more effective dose selection for regulatory studies.

Collaboration with external scientific groups and organisations enables us to explore the use of developing technologies and capabilities such as proteomics and metabolomics platforms, allowing us to extend data sets further from within an individual study.

Data from studies may be included in peer review publications or presented at internal and external meetings and conferences to share new knowledge and learning in the wider context of drug safety assessment and development.

Species and numbers of animals expected to be used

- Rats: 9000
- Mice: 3500

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



Studies will be conducted by dosing routes similar to those used in man, e.g. by mouth (orally) or by injection. The animals are then observed regularly to monitor changes in appearance and behaviour. Procedures carried out during these studies include:

Weighing: as a loss in body weight is often an early sign of harmful effects in animals

Blood sampling or collection of urine for measurement of different components, as changes in these may serve as early indicators of toxicity. Doctors, for similar reasons, often take blood and urine samples from humans.

Electrocardiography (ECG) monitoring to assess changes in heart function (e.g. number of heartbeats per minute). This technique is also used by doctors to assess heart function in humans.

Animals may undergo surgical procedures, for example to implant telemetry devices for non-invasive, longer term, cardiovascular monitoring; for implantation of vascular cannulae, or for non-recovery investigations

A degree of restraint or confinement may be required for some of the various dosing, sampling or assessment procedures such as those required to assess motor co-ordination (balance), muscular strength (ability to grip) or visual assessments.

At the end of the study, the animals are humanely killed by an overdose of anaesthetic. Where appropriate animals may be re-used for subsequent investigations. Samples of various organs are taken and examined under a microscope to ascertain whether the potential new medicine has caused changes that would rule out administration to humans.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

From experience, some animals are expected to have mild adverse effects such as slight weight loss. Animals may show more significant (moderate) adverse effects e.g. more marked weight loss, or changes in appearance (e.g. ruffled fur in rodents) or behaviour (e.g. reduced activity) indicative of moderate severity. Humane end-points are applied, under veterinary guidance as necessary.

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

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

The maximum severity for both mice and rats is expected to be moderate. It is estimated <70% of animals will reach moderate severity, with the remainder reaching either mild severity or being non-recovery in nature.

What will happen to the animals at the end of the study?

- 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?

Prior to studies within this programme of work, a number of in vitro (in cells) and/or computer programme simulated tests will have been used to establish early safety liabilities and risk associated with a target or novel agent. Only by using animals as part of a hypothesis-driven experimental design can we evaluate the impact of these in vitro /simulated findings on disordered physiological processes and pharmacology in a whole animal system, and ultimately the potential for adverse effects in humans.

What was your strategy for searching for non-animal alternatives?

We are continually investigating alternative in vitro systems that may be utilised to assess certain end points and thus reduce the number of in vivo studies required. For example, specific types of hazards, predictive of potential to changes in blood pressure and heart rate, can be detected using in vitro systems and these assays are performed early in the safety assessment programme. "Organ-on-Chip" technology is extensively used, particularly to understand liver and bone marrow toxicology. These are self-contained units containing living human cells. The human cells recreate the physiological functions of organs without using animal models.

Why were they not suitable?

In silico and in vitro approaches are becoming increasingly powerful tools in the design and development of new medicines and are used extensively to understand potential adverse effects. These approaches can help in understanding various isolated aspects of a new drug's safety profile and these methods are used to screen out those that clearly do not have the desired properties. However, because the safety profile for a given drug is multifactorial, it is not always possible to mimic the entire breadth of physiological responses and any interactions as seen in a whole animal (in vivo) system and to this end, such studies performed under this licence remain 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 numbers for all protocols have been calculated on an estimated workload derived from the previous tenure of this licence over a 4.5 yr period. These numbers are not definitive and could change in relation to the target/therapeutic disease area needs.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The numbers of animals used for each experiment will be set after consultation with a professional statistician to ensure all animal studies use the most appropriate design to



answer the primary objective and to avoid bias. Animal numbers are kept to the minimum commensurate with meeting the objectives, and the endpoints being measured.

What other measures apart from good experimental design will you use to minimise numbers?

The following approaches will optimise the number of animals used, obviating further studies being performed as stand-alone studies under this licence:

Where possible, additional sampling (e.g of tissues at the end of a study) may be included in planned studies thus deriving more data from one experiment.

Samples may often be stored awaiting the development of a suitable assay, for examples the use of samples to obtain a measure which is a similar measure to that used in patients.

Where possible, control or vehicle-dosed tissue may also be used for other experiments.

If a new technology or concept is to be used within this Project, small pilot studies will first be performed to validate equipment and gain confidence, expertise and experience, prior to committing to larger studies.

Studies employing a dose escalation design will be staggered to prevent unnecessary suffering and animal losses; once maximum tolerated doses are reached, further dosing will cease.

Other steps taken to ensure minimal animal use include the use of databases and computer modelling. Data generated under this project will be uploaded to a corporate database. This is critical to reducing the number of animals used and allows:

Rapid access to data by all scientists and avoids requests and repetition of studies.

Other research scientists carrying out evaluation of the same test compound may design their studies at appropriate dose levels, routes etc.

The refinement of computer models to predict pharmacokinetic (PK) properties utilises software to simulate multiple dose PK (level of drug in the blood or tissue), rather than repeat dosing animals, and is critical to selection of appropriate doses for toxicological evaluation.

Other approaches that reduce animal numbers used include:

Crossover study design may enable all dose phases to be performed in one animal – typically used for studies measuring heart rate and blood pressure where re-use enables the same animals to be used for multiple evaluations.

Increased assay sensitivity and the development of microsampling has enabled serial sampling in mice where full PK profiles are generated in single animals rather than the multi animal approach utilising 3 animals per time point. This approach reduces animal numbers used and reduces data variability across cohorts.

Refinement



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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The species, models and methods used within this licence are widely accepted and have a proven record in generating translatable preclinical safety data. There is a wide knowledge of the response of rats and mice to various chemical entities and a wealth of background safety data in the pharmaceutical industry. Typically, the rat is the rodent species of choice unless it is known to be an inappropriate model for man for the test substance or target-related toxicity. Mice may be used where they are considered a more appropriate species than rats e.g. for kinetic reasons and/or presence of pharmacological target.

The scientific objective of this Project is to assess the early safety risk of novel medicines. To enable this assessment, agents will be administered at doses that may result in moderate bodyweight loss and clinical signs. The severity limits are those considered to be the minimum commensurate with achieving the study objectives. In all studies conducted under the authority of this licence, the upper severity limit will be moderate.

Blood volume taken for measures will be minimised by using microsampling techniques which significantly reduce the volume of blood removed from each animal, but still produce high quality data.

Best practice, for example the use analgesics after surgical procedures will be employed to minimise suffering.

Adverse effects will be kept to the minimum to achieve the objectives. Animals will generally be housed in groups and provided with specific materials to provide environmental enrichment.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Generally, the rat is the rodent species of choice unless it is known to inaccurately reflect man for a particular compound. There is a wide knowledge of the response of rats to various chemical entities and the translatability of these responses to man is more defined in the rat than other species due to a wealth of background data in the pharmaceutical industry. Rats are big enough to provide repeated blood samples for toxicokinetics, thus requiring significantly fewer rats than mice to achieve the same objective. However, mice may be used where they are considered a more appropriate species than rats e.g. for kinetic reasons and/or the presence of the pharmacological target.

As part of safety assessment profiling a molecule may be assessed for effects on complex functional / behavioural observations such as visual acuity, co-ordination / CNS effects or cardiovascular parameters in telemetered animals. These tests evoke responses in rats and mice that can be readily translated to man and the clinic. These assessments are more difficult in species that display more limited behavioural repertoires.



Studies are performed in animals that are terminally anaesthetised, where appropriate. However, many toxicological responses and pathologies evolve over time with repeat dosing.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Where possible, all animals will be group housed. The use of the REDACTED for rodent infusion work enables group housing of surgically cannulated rats between infusion cycles where previously this was not possible.

The majority of rats are housed in cages which offer a diverse environment divided over two levels, which encourages natural rat behaviours such as “rearing” and gives a greater floor area for exploration, AND ARE provided with specific materials to provide enrichment, including a selection of nesting materials, chew blocks, refuges/hiding places and foraging. All animals will receive a minimum of 7 days acclimatisation before use in experiments.

We are fully committed to NC3R recommendations for single needle use for parenterally (non-oral) administered substances ensuring that undue pain and suffering linked to the use of blunt needles is not experienced. As described above we are also heavily involved in non-aversive handling techniques for mice and have begun to ensure this is applied as “best practice”. Training regimes to acclimate mice to “cupping/tube handling” techniques are underway to reduce stress in mice and therefore improve the integrity of data generated.

Blood volumes taken for measurements will be minimised by using microsampling techniques which significantly reduce the volume of blood removed from each animal, but still produce high quality data.

Adverse effects will be kept to the minimum to achieve the objectives, and will be closely and regularly monitored during and after regulated procedures.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Best practice, for example the use of analgesics after surgical procedures will be employed to minimise suffering.

FELASA guidelines are used as a baseline to ensure experiments are conducted in the most refined way. Historically, guidelines such as those produced by FELASA have been used to define the upper limit for bodyweight loss at 20% in safety assessment studies. Our organisation has actively contributed to a global collaboration that used evidence to define the upper limit for body weight loss in maximum tolerated dose (MTD) studies (conducted at contract research organisations). We have extended this guidance to studies run in-house under this and previous Project Licences, meaning that a gradual bodyweight loss limit compared with baseline (pre-dose) and/or age matched controls, will be commonly less than 15% over a period of one week.

The dose selection principles outlined in the LASA/NC3Rs guidance on dose selection for pharmaceuticals will be used where appropriate.



How will you ensure you continue to use the most refined methods during the lifetime of this project?

Advances in the 3Rs are regularly shared throughout our organisation and establishment network via e- mail or the internal intranet system, as part of the named person structure implemented by the Act (NTCO, NVS, NIO, HOLC, PIL, PLH).

Specific 3Rs based objectives are regularly included as part of an individual's yearly performance goals and are assessed as part of that individual's performance. In addition to this, we promote an annual presence at industry-focused meetings (IAT, FELASA) with the sole purpose of attaining and sharing 3Rs based knowledge with other companies and institutions. The "cupping/tube handling" technique for mouse handling is such a technique that has been introduced from these activities and is currently being implemented, validated and reviewed across our global sites as part of our continuous commitment to animal welfare improvements. One of the functions of this licence application is to enable the introduction and review of new techniques or technologies that may be applicable to the 3Rs. The use of the REDACTED for rodent infusion work enabled group housing of surgically cannulated rats and is one example of this protocol in use.

Explain the choice of species and the related life stages

Rats and mice have a well characterised pathophysiology, and are a widely accepted species to measure safety/toxicity effects.





81. In vivo profiling of novel compounds

Project duration

5 years 0 months

Project purpose

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

Key words

pharmacokinetics

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The overall aim of the project is to give support to the discovery phase of research projects for external clients, ensuring that potential medicines with suitable pharmacokinetic – what the body does to the drug (PK) and pharmacodynamics – what the drug does to the body (PD) properties can be selected for further development to treat human disease effectively. PK is investigated by studying how potential medicines are absorbed and distributed in the body as well as how they are broken down (metabolised) and excreted. PD is investigated by the affect a compound has on the cardiovascular system

Typically a medicine that is given orally is dissolved in the gut, absorbed into the blood and then the circulated around the body. It may be metabolised, usually in the liver, and it, and/or its break down products (metabolites) excreted in urine and faeces. Some medicines cannot be given orally: for example, due to poor absorption from, or breakdown in, the gut and another route must be used. Other medicines, such as those given by inhalation, do not need to go into the blood stream in order to elicit the desired pharmacological response and/or are more effective when directly delivered to diseased tissue. Confidence in predicting pharmacokinetic properties in man is gained by studying the action of the potential medicine in more than one animal species.

The information gained from studies carried out under this licence will help to;

Understand and then improve the way the compound is given so that there is sufficient information available to treat the disease effectively.



Understand and then improve the length of action of the compound so that it is likely to stay in the body long enough when given in a dosing routine that is easy for patients to use. Potential clients are required to disclose any previous in vivo investigations done on their compounds, to avoid any unjustified duplication of procedures.

To achieve these goals animals will be dosed, usually only once, with the candidate medicine and then small blood samples taken in order to assess how the candidate medicine's concentration changes over time and whether this affects its ability to treat a disease.

Dose concentrations will be kept as low as possible to ensure any adverse effects associated with the candidate medicine are minimised. Blood sampling methods will favour the least invasive possible to minimise any distress associated with taking blood samples.

Whilst we cannot predict unforeseen circumstances, but we only anticipate 'mild' to 'moderate' levels of severity in the licence.

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

What are the potential benefits that will derive from this project?

Work under this licence will assist our clients in selecting compounds with an expectation of suitable properties in man and thus reduce the risk of exposure of human volunteers and patients to compounds that would be unsuitable as therapies. The work will enable clear decisions by our clients to progress or halt compounds at key project milestones. Data generated will assist in designing appropriate regimens and limit unnecessary use of animals for pharmacological and toxicological studies.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Rats, mice and guinea-pigs will be used on this licence. It is expected that no more than 11250 rats, 12300 mice and 600 guinea-pigs will be used over the life of the licence.

Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Adverse effects (eg piloerection, mild sedation and salivation) are those associated with routine routes of administration, sampling and those associated with general anaesthetic. In these cases, the likelihood of occurrence is estimated at <1%. The administration of test compounds/ substances will have the potential to affect all animals. Close monitoring and



use of pilot studies, will help to keep the incidence of adverse effects to a minimum. Animals will be humanely killed at the end of all protocols.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

There are no non animal tests (in vitro - in glass) that completely mimic and predict many key aspects of the work described in this licence eg: does the drug get to the target tissue or organ and does it affect blood vessels. These aspects are often the result of the interaction of many individual biological processes and these interactions cannot be reproduced by in vitro (non animal) alternatives. For most organs there are currently no in vitro models that predict organ clearance and only an intact animal can provide an adequate integrated system. Consequently, animal testing is essential to achieving the overall objective of this licence.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Certain properties of drugs can be studied using a range of in vitro ('in glass' or 'test tube') and in vivo ('in life' or 'in animals') studies. It is standard practice to conduct the major proportion of testing in vitro using cells and tissues obtained from humans or animals. This strategy makes a vital contribution towards minimizing animal usage. In our experience, fewer than 10% of compounds tested in vitro are progressed to in vivo studies. In addition to in vitro testing, the properties of compounds are also predicted based on knowledge of their structure ('in silico'). Whilst in silico (using computers) predictions or in vitro studies can provide a wealth of information on individual body systems, eventually studies in living animals are needed. Animals offer suitable models to replicate the interplay between different processes that can influence the disposition (what happens after it is given to the patient) of the potential medicine. Once in vivo work is deemed necessary, there are a number of approaches adopted in order to minimise the numbers of animals used;

Typically group size is 3 rats

Refinements in sampling in mice, reducing overall numbers required.

Consideration of statistical analysis, to use the smallest group sizes possible, yet maintain adequate precision.

In pharmacodynamic studies the minimum number of animals required to detect effects of scientific interest will be determined using sample size calculations.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Purpose bred, adult free living animals of assured health and genetic status will be obtained from commercial suppliers or from breeding colonies. Most studies will be conducted in rats; this is supported by in vitro testing which shows the relevance of this species to man. It is necessary to use other rodent species, for the purpose of this project



mouse and guinea-pig if these species are more relevant to man for a particular research project or if more than one species is needed to build further confidence in predicting to man.

Animal suffering will be minimised by the following;

Competent personnel will perform all studies on this project licence and adverse effects resulting from regulated procedures will be minimised by careful handling and the application of good technique.

Guidelines on the limit of volumes of administration of substances and blood sampling will be strictly adhered to.

A refinement in sample analysis has led to the reduction in total blood volumes required, now typically 20µl sample size. Thus reducing the burden further.





82. Lymphocyte development and immune function in the tissues

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims: 0

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The aim of this project is to understand the blood cells that enter and populate various tissues (e.g. lung, pancreas, kidney, etc.). We seek to understand the pathways that control how many there are and the signalling that is necessary to get there and reside long term. Additionally, we seek to understand the function that tissue-resident cells may have in both homeostatic conditions that may be involved in development, maturation and ageing of various tissues and the involvement of these cells in disease onset, progression and resolution.

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

Why is it important to undertake this work?

Intensive research over decades has given us a comprehensive understanding of the components of the immune system and their function in health and disease. However almost all of this research has taken place on circulatory cells or cells in secondary lymphoid tissues: blood cells or accessible tissues such as tonsils in humans, and blood,



spleen or lymph nodes in mice. Arguably, however, the most important immune reactions are those that occur in the tissues: asthma, allergy, most autoimmune disorders, many autoinflammatory disorders, most infections and most cancers are diseases of the tissues. It is therefore critical that we start to understand what is different about the immunology of the tissues compared to the immunology of circulation: what controls homeostasis, what controls development, what are the kinetics, what are shared and distinct functions.

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

Key output of new information: a comprehensive understanding of the cellular and molecular basis of tissue lymphocyte biology, with regards to homeostasis (the number of cells in the population), development, migration and function.

Key output of new genetic tools: the generation of genetically modified mouse strains is to enable proliferation and apoptosis (cell death) quantification, tracing and modification of tissue-resident migratory populations, and tissue-selective expansion/deletion of lymphocyte subsets.

Key output of resources: we will create online resources for the major datasets generated through this study

Key output of publications: we will seek to publish all results from this study

Key output of patents: where recommended by our knowledge exchange and commercialisation team we will patent findings.

Key output of products: this project will develop immunomodulatory reagents that modify the immune context of specific organs. These products may have potential for clinical application.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Other Researchers:

Our work will create a unique resource of systematic transcriptomics and functional biology of tissue- resident lymphocytes. This resource, through both publication and the provision of online interactive datasets, will directly inform the research of other researchers. The work will identify candidate pathways for investigation in biomedical research, and in silico data mining resources to substitute for animal research. Moreover, researchers will be able to use the genetically altered mice that we generate and characterise.

Industry:

Tissue-specific immune modulation remains the key objective of many drug development programs. Targeting systemic pathways not only reduces efficacy when treating organ-specific disease, but it increases the chance of unwanted side-effects, from systemic immunosuppression to off-target inflammation. Tissue-specific targeting, by contrast, increases efficacy and reduces unwanted side-effects. By providing a functionally validated list of genetic and epigenetic pathways that can modulate immunity in a tissue-specific manner, and potentially pre-clinical drugs that work in this manner, we will e development of tissue-specific immune modulation in industry.



Patients and Clinicians:

We will continue to ensure that our research findings in mouse models are translated to the human immunology context. >50% of our research publications in the past 5 years have included data derived from human samples, ensuring, wherever possible, that our results are relevant cross-species and result in translational advances. REDACTED allowing us to directly work towards translating this pre-clinical research tool into clinical practice.

How will you maximise the outputs of your work?

We will publish all data and also create online data resources of the complete dataset. This will enable other researchers to access and mine our data for incidental findings that we did not identify. We will share our research tools, methods developed and datasets freely. We will patent findings when advised that this approach will increase uptake by industrial partners.

Species and numbers of animals expected to be used

Mice: 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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

In protocols 1-4, we will perform the injections and surgeries required to generate new mouse models. These surgeries are similar in nature to those involved in IVF, and while the surgery does result in discomfort, pathologies are generally avoided.

In protocols 5-8, we will breed and maintain mouse strains for experimentation. Protocols 5-6 will cover maintenance until one year of age, covering most mice used in this study. A small subset of mice will be aged out past 1 year, as some immunological processes change with age and this needs to be accounted for. Protocols 5 and 7 cover the maintenance of mouse strains where no or mild adverse effects are predicted. Protocols 6 and 8 cover the maintenance of mouse strains where a fraction of mice are expected to have adverse effects that are moderate.

In protocol 9, we will inject mice with substances to modify their immune system, using ex vivo analysis.

In protocol 10, we will create bone-marrow chimeras. This is a procedure very similar to bone-marrow transplantation in humans, and while it results in discomfort and some adverse effects, pathologies are generally avoided.

In protocols 11-13, pathologies will be induced. Protocol 11 involves giving mice an attenuated flu virus or a dead vaccine / synthetic immunogen. This results in some inflammation, similar in duration and severity to that of a patient receiving a vaccination.



This procedure was selected due to the physiological relevance of flu vaccination to humans, as well as the highly-refined low-pathology nature of the protocol in comparison to other respiratory infection models. Protocol 12 will induce colitis, which is a pathology of the gastrointestinal tract. This will result in moderately severe discomfort for the mice, with diarrhoea and weight loss similar to a patient with Crohn's disease or ulcerative colitis, generally for a period of around a week. This procedure was selected as it is highly validated as a read-out for immune function, allowing us to reduce total mouse use. Protocol 13 will induce allergic asthma. This will result in moderately severe discomfort for the mice, with breathing difficulties similar to an asthmatic patient for several weeks. The model was selected as a non-infectious counterpart to the flu model (to determine whether there are differences in gene functions in infectious vs non-infectious inflammation) due to the highly-refined low-pathology nature of the protocol in comparison to other respiratory inflammation models.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The majority of mice in this project will experience no or minor adverse effects, similar to the transient pain and inflammation caused by an injection. A minority of mice in this project will experience adverse effects that are rated as moderate. In most cases these are transient effects, with pain, weight-loss, discomfort or minor diarrhoea, similar to recovery from a standard surgical procedure and lasting several days. Other mice will have moderate adverse effects from disease induction, with exposure to attenuated influenza (similar to a human cold in severity), induction of colitis (similar to the human disease of colitis, with intermittent gastrointestinal discomfort over the course of around a week), and induction of allergic asthma (similar to human asthma, with intermittent restriction in lung function over the course of several weeks).

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

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

Total animals (mice) used: 59109

Total animals used (mild phenotype): 53438 (majority >80 will be due to tissue biopsy for identification purposes)

Total animals used (moderate phenotype): 4250

What will happen to the animals at the end of the study?

- 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?



Immune responses are complex processes involving multiple cell types and interaction with the tissue microenvironment. When tissue-resident immunity is involved, further complexity is generated, with modifications to the system in primary lymphoid tissues, secondary lymphoid tissues, circulation and the tissues. To date, an in vitro model of such a complex system is not available, and it will not be possible to develop such a model until we have a full understanding of the in vivo context we seek to model. The complex interactions between different cells of the immune system cannot be modelled adequately in tissue culture or by computational methods. This is because immune cells are highly sensitive to the environment, responding to cellular, extra-cellular matrix and soluble mediator cues, which differ in minute but important ways between tissues, between locations within tissues and even temporally within the same tissue location. Furthermore, even the successful modelling of a single anatomical location (a scientific feat not yet possible) would negate the highly migratory nature of lymphocytes, which differentiate and activate in multiple tissues, linked by complex blood and lymphatic migratory patterns. Ultimately, while we can model simple distinct processes in vitro, complex immune reactions need to be modelled in vivo.

What was your strategy for searching for non-animal alternatives?

We have considered the use of in silico modelling, cell line work, in vitro cultures, organoid systems and non-vertebrate animals.

Why were they not suitable?

For in silico modelling, there are no datasets currently available that cover the experimental designs needed. A key output of this project will be the generation of these datasets and in silico models. For cell line and in vitro culture work, the systems do not adequately recapitulate the complexities of immune regulation. Certain validation experiments can and will be performed in cell lines and in vitro, however the in vivo experiments described here cannot be performed with the same degree of scientific accuracy without an in vivo system. Organoid systems are showing increasingly interesting results at modelling single anatomical sites, however the study of immune responses in organoids is limited by the need for primary MHC-matched donor cells and the lack of normal vascularisation. Added to this is the multi-organ nature of the questions here being assessed. Non-vertebrate animals do not have adaptive immune responses and therefore cannot be used in this project.

Reduction

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

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

The numbers of mice required for the generation of modified mouse strains are based on the standard operating procedures extensive experience and literature review. The numbers of mice required for the breeding and maintenance protocols is based on estimations of mouse strain numbers, experience at sustainable colony management practice and the frequency of required genotype combinations. The numbers of mice required for individual experiments are based on power calculations and statistical



modelling. We input the known statistical properties (phenotype average and variation), decide upon the minimal effect size acceptable from the experiment from a biological perspective and hence calculate the appropriate group size. The number of experiments required within each protocol is based on the assumption of all go-no go decisions being positive and successful grant funding achieved.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The key steps taken to reduce the number of animals being used are: 1) use of controlled animal facilities to reduce biological and environmental variation, 2) use of optimised internal standard operating procedures to reduce technical variation, 3) consultation with a full-time mathematician embedded in our research group, 4) design of experiments to allow large-scale measurements from each individual mice (e.g., parallel assessment of lymphocytes inside every tissue per mouse, rather than running one experiment for the lung, one of the gut, etc), 5) design of multiplexing screening experiments, such as CrispR screening, where many candidate genes can be functionally tested in the same mouse.

What other measures apart from good experimental design will you use to minimise numbers?

Where possible we will share mouse strains rather than generate new strains. This bidirectional exchange will reduce net mouse use during mouse generation. Breeding strategies are designed to minimise the number of mice experiencing mild or moderate severity, e.g., by only bringing together harmful combinations of genetic modifications when required for experiments, rather than constantly maintaining the combination. We have a dedicated mathematician embedded in the group to generate mathematical models from the data that can guide future experiments with greater levels of precision. We will stock frozen stores of biological samples (bone-marrow, serum, tissue sections) so that certain experiments can be performed on previous samples rather than using new mice.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice are proposed as their immunological responses bear a high degree of similarity to that of humans. The genetic background and immunoreactivity of mice is very well characterised and there are a wealth of reagents and research tools available that are compatible with mice.

Suffering will be minimised by provision of analgesia where appropriate, provision of diet-gel food on cage floors should animals have difficulty accessing water due to mobility impairment, sub-cutaneous hydration if dehydration is apparent, housing in heat room/on heat pad if temperature drops significantly (e.g. following anaesthesia).



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The choice of species is limited by the fact that invertebrate species do not have an adaptive immune system that is comparable to humans. Mammals share key aspects of immune biology with humans, and have formed the basis of the modern immunological synthesis. Immune responses change markedly with age, and thus studying adult mice is the appropriate life stage. Terminal anaesthesia is used when experiments are of sufficiently short-term (minutes to hours) to allow for it, however when the immune processes to be study are in the range of days to weeks terminal anaesthesia is not feasible.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Given the impact on the well-being of the animals, this project has carefully selected protocols that are only mild or moderate, avoiding any studies classified as severe. Experimental pipelines work first through the protocols that are considered mild, with only high priority experiments being performed in the moderate models. For the moderate severity models, our work focuses on identifying the onset of pathology and key pathological stages, or delaying the onset of pathology or key pathological changes. As we are not studying the effects of chronic pathology or trying to treat chronic pathology we can avoid any prolonged (> weeks) adverse effects to mice. Additionally, input will be encouraged from animal technicians and NACWOs (Named Animal Care & Welfare Officer, responsible for overseeing the day-to-day husbandry, care and welfare of the protected animals held at their establishment) following studies to identify possible areas for refinement. Clinical score sheets linked to humane end points and cumulative severity limits will be developed in collaboration with REDACTED staff and put in place for all diseases models.

Administration of compounds is performed according to the route and dose that minimises toxic effects.

All recovery and long-term non-recovery surgery will be done aseptically to HO Minimum Standards of Aseptic Surgery. Peri-operative and post-operative analgesia will be given when necessary using advice from the NACWO and NVS (Named Veterinary Surgeon, responsible for, monitors and provides advice on the health, welfare and treatment of animals). Choice of analgesic, duration and dose will be adjusted to the clinical signs observed taking into account possible impacts on the experimental plan.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

All experiments which will integrate refinements from the NC3Rs (e.g., the ARRIVE guidelines), the LASA aseptic guidelines, LASA Diehl guidelines on volumes and frequency limits and the most up-to-date veterinary knowledge.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will actively stay updated with our field of research through collaboration, conference attendance and reading the literature. We will take particular note of any technical



advances that enable reduction, refinement or replacement in our experimental design. The local mouse facility is also a key source of knowledge, transmitting the latest information on the 3Rs to researchers. Internal protocols are shared across the institute, enabling rapid uptake of any improvements to the method across groups.

Explain the choice of species and the related life stages

We are using mice because they are currently the best non-primate model for human immunology. The immune system comparison of mouse and humans shows strong parallels between cell types and functions, in both health and disease, although some details vary across the species. For any other mammalian species, the level of basic immunology knowledge is much lower, and an enormous amount of validation work would need to be run before the project could be initiated. Furthermore, many tools are already developed for mouse work, including optimised immunomodulatory drugs and processes for genetic modification.





83. Maintenance of Genome Stability in Development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

We aim to understand the mechanisms which detect and repair damage to the DNA (that contains the instructions to make an organism) between generations. To understand how mutations are avoided or transmitted from one individual to its offspring and to understand the DNA repair mechanism that are specific to the cells responsible for heredity.

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

Why is it important to undertake this work?

Failure to repair DNA damage can either lead to cell death or to errors in the genetic instructions (known as mutations) that built cells and organisms. In this project we aim to gain insight into how DNA damage is detected and repaired accurately. In order to understand how damage and repair are affected by general processes such as metabolism or development we will use animals since none of these functions of the body can be recapitulated by cell cultures or in vitro methods. Ultimately we aim to understand how the genetic information is passed from one generation to the next with sufficient accuracy to sustain normal development, avoiding genetically transmitted disease and many childhood cancers.



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

Results obtained from this work will be published in a timely manner

We will define the DNA repair requirements necessary to make functional germ cells

We provide new insight into when during germ cell development mutations arise

We expect to identify new factors ensuring the quality control of germ cells

We expect to elucidate which DNA repair pathways are responsible for tissue specific mutation

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Throughout project

The results obtained will be communicated to other researchers at national and international conferences and in academic publications. The results of this project are likely to promote our understanding of how the genome of germ cells are maintained free from errors. This will include the identification of new components and new cellular targets.

Long term

This project will increase our fundamental understanding of how genome evolution occurs. Mutations which arise in the genome adversely affect human health as this is the process by which genetic diseases arise. This is also important to improve our understanding of infertility as damage to the gametes is an important cause of infertility. In the long term understanding the sources and consequences of damage to the gametes may allow us to identify damaged gametes or devise interventions which may prevent damage to the gametes in the first place.

How will you maximise the outputs of your work?

Animal models generated in the licence will be made freely available to academic researchers.

Results will be published in a timely manner and made freely available following MRC guidance(Open Access).

Data sets generated from mutational analysis will be deposited in a public repository

Species and numbers of animals expected to be used

- Mice: 39875

Predicted harms

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



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The vast majority of mice used in this licence (>90%) will not undergo any intervention. They will be used in breeding or killed and tissues obtained. This means that those animals will not suffer any harm due to the scientific procedure.

More, rarely mice will have interventions. Of those the majority will receive a single injection which in itself will cause transient pain. The mice are expected to make a full and uneventful recovery.

We may perform surgery on mice to allow us to put cells into the testis. These mice will have anaesthetic during the procedure and be given pain relief when they wake up. During the surgery an incision will be made in the scrotum and the needle placed into the testis. The wound will then either be glued or sown closed. Mice are expected to make a full recovery and not to experience any suffering when the wound is healed.

We may also use surgery to place a small pellet under the skin of the mice. This is to allow us to give the animal a drug. These mice will have anaesthetic during the procedure and be given pain relief when they wake up. During the surgery an incision will be made in the skin. The wound will usually be glued closed. The mice will make a full recovery and will not suffer when the wound is healed.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We will generate mice that lack the ability to repair damage to their DNA or cause damage to their DNA. These mice may have a predisposition to cancer. The best indicator of either of these diseases is weight loss – therefore mice with a genotype that predisposes to these diseases are identified early. We will carefully monitor those mice and cull them before they suffer any ill health when possible.

The new technology known as ‘CRISPR-Cas9’ provides us with a new way to generate mice that lack the ability to repair DNA damage. This means that we make alterations to the DNA of the mouse when it is one cell (straight after fertilization). We then use surgery to put that embryo into a female so that it can continue to develop and be born.

One of the methods we will use to generate mice will involve delivering foreign DNA into their liver cells. We will do this by injecting a solution (containing the foreign DNA) into their vein. In order for the DNA to get into the cells we must inject it in a large volume over a short period of time. This causes the mice to temporarily lose consciousness, however we anticipate that all mice that undergo this procedure will be under anaesthetic. The mice rapidly recover (5-30minutes) after which this procedure does not cause additional suffering of the mice.

We will perform bone marrow transplants and also transplantation of cells into the testes during this project. In order to achieve this we must prepare the recipients by conditioning – the same procedure is performed in humans before transplantation. As in the case of humans conditioning will involve giving mice chemotherapy (used in the treatment of human cancer) or exposing them to X-rays (used when treated certain cancers or autoimmune diseases). This may result in mice losing weight for up to 2-3 weeks before



recovering. If mice do not recover then they will be culled before they suffer any additional ill health.

Finally, in order for us to really assess the repair of specific kinds of damage to the DNA of the germ cells it will be necessary to expose mice to known DNA damaging agents. Following this process we anticipate that approximately 70% of the mice may lose weight but they will be carefully monitored. Weight loss is a good indicator of the health of the animals. Animals that gradually lose weight will not be allowed to lose more than 20% of their body weight if other clinical signs are seen. If no other signs are seen animals may be monitored for a further 48hrs before they are killed only if their weight remains stable and no other signs develop.

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

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

The vast majority of mice are not expected to suffer because of this project (>90%).

A small proportion of mice will develop cancer and those mice will lose weight before being killed.

Mice that undergo surgery will suffer from minor and transient discomfort.

Mice that receive conditioning for either bone marrow or testis stem cell transplants or that are given DNA damaging agents will lose weight lasting less than 3 weeks before beginning to put weight back on.

What will happen to the animals at the end of the study?

- 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 very difficult to study the production of sperm and eggs in any system other than a whole organism. This is because sperm and eggs cannot divide to produce more sperm and eggs (since they only hold half of the amount of genetic information as all other cells). Non-animal models, whilst of huge promise, remain of restricted value.

We also want to see how these cells begin life in an embryo and this is not possible to achieve without using animals.

Mammals are the most appropriate model because the way in which they make their germ cells is distinct from other vertebrates. We are particularly interested in the mammalian germ cells as they go through the same set of processes as human germ cells.

What was your strategy for searching for non-animal alternatives?



We will perform initial experiments by growing these cells in a test tube. However, this has limitations as the process of maturing germ cells in a test tube is distinctly different than what happens in an organism.

Why were they not suitable?

The test tube germ cells do not undergo some processes that happen in a live animal. These processes can produce DNA damage, therefore we cannot always use this system to study the process of repair.

We can provide a first hand example of why they are not suitable. We employed our non-animal system to study the role of Ercc1 (a DNA repair factor) in embryonic germ cell development. The phenotype we observed in the non-animal system was very different from the results obtained in mice (the mouse phenotype is similar to what is found in human patients). Therefore, whilst 25% of my lab tries to develop non-animal systems these are not yet at a stage to replace the use of animals. The reason for this that this problem remains complicated. However, the advent of systems to study human germ cell development show enormous promise. It is my sincere hope that over the next decade we will vastly reduce our reliance upon animal models to study embryonic germ cell 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 vast majority of mice used in this project are required to generate new GA mice or combine mice with multiple alleles. As we work on fertility the maintenance of these mice is complex as they often cannot have young. Our experience over that last 12 years plays a significant part in estimating the numbers of mice that will be used in this project.

For many other aspects of this project the aim is to generate mutagenised DNA. As such the emphasis is on production rather than an in vivo experiment. The minimal numbers of mice are used in order to obtain that material. We have sufficient experience to know the efficiency of transplantation or hydrodynamic delivery to be able to use the minimal numbers of mice to successfully obtain sufficient amounts of mutated DNA.

For individual experiments we do use power calculations to determine the numbers of mice needed. This can be challenging as we use embryos and so litter size (which is variable) complicates our calculations. In these occasions we need to make an estimate of the effect size. We determine this using a surrogate marker or by performing pilot studies.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Experiments designed in this project are relatively simple. We have sufficient in house statistical knowledge to be able to plan those experiments. Typically we want to ask how



many pregnant females are required in order to determine the frequency of primordial germ cells at developmental day E9.5. We either rely upon data from our in vitro assay or a pilot experiment to estimate the magnitude of reduction and to estimate the Mendelian frequency. We then use historic data of litter size at E9.5. Together these data allow us to calculate how many pregnant females are required. Animals are retrospectively genotyped.

What other measures apart from good experimental design will you use to minimise numbers?

For conventional breeding we have a number of tools to ensure efficiency. These range from frequent reports of breeding efficiency to the use of conditional alleles to circumvent the sterility of certain mice.

The use of CRISPR-Cas9 technology provides us with the potential opportunity to reduce the number of mice used when generating new genetically altered mouse strains. Firstly, we can reduce the numbers of mice that undergo the superovulation procedure. Secondly, there is a reduction in the subsequent breeding. Finally, we can generate mice that have multiple genetic changes at the same time. To do this by breeding would involve multiple generations and the use of large number of mice.

We also aim to use a technique known as 'hydrodynamic gene delivery'. This allows us to generate an experimental animal that can be used without the need for extensive breeding. This will result in us using fewer mice in order to conduct this specific kind of experiment. We may use inactivated viruses to help us delivery the right amount of DNA to the liver cells.

Finally, we will cryopreserve our strains. This prevents us needing to keep all of our mouse strains alive just to perpetuate the strain.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Many of the models used in this project lack the ability to fix damage to DNA (the blueprint of life). When possible we will maintain animals so that they have one working copy of the DNA repair factor. This means that we will only use mice that lack the DNA repair factor when we do the experiment.

Some DNA repair factors are important for life. However, we are only interested in studying what happens in germ cells (the cells that give rise to eggs and sperm). Therefore instead of working with mice that lack the DNA repair factor everywhere (and show signs of suffering) we will make lack the repair factor just in the germ cells. This allows us to study the process we are interested causing as little distress to the animals as possible.



Exposure to agents which cause damage to the genome cause suffering. We minimise this by using the lowest possible dose that still allows us to obtain useful information. We are guided by published results and attempt at all times to minimise the suffering to the animal. An example of this is exposure to irradiation which we split between two doses to improve the outcome for the animals undergoing the procedure.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The majority of the animals that we use for experimental reasons will be used during embryonic development. This means that only embryos that lack the DNA repair factor will be generated. This means that we can reduce the number of adults born that lack the repair factor which may cause distress.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Many of the mouse models used will employ inducible constructs when possible. These mice should not display a phenotype until candidate gene expression or deletion is induced. This will allow us to study the effect of deletion in the germline without causing harmful non-germline phenotypes.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We refer to the NC3R website as a great resource for ensuring that we are using the most refined experiments. In the case of surgical and tumour models we follow the current guidance and check the NC3R website to ensure that this is up to date. We also think with the ARRIVE and prepare guidelines when planning and also experiments to ensure that our experience can be communicated to other researchers (PREPARE: guidelines for planning animal research and testing. Smith AJ et al). Surgery will be performed to best practice as laid out in the LASA guidelines (http://lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf). When substance are administer or blood samples taken best practice will be followed as set out in A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes, Diehl et al.. When planning experiments we will refer to Norecopa (<https://norecopa.no/>) to identify guidelines, search for alternatives and ensure that we are following best practice.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Over the course of this project we will keep up to date with the latest advances in the 3R. Firstly, our named information officer provides us with regular emails outlining both interesting findings but also potential training opportunities. Secondly, we have a close relationship the with animal technicians that provide daily care of the mice. The technicians regularly undertake formal courses (IAT) and attend international conferences. They therefore act as an invaluable source of information. They frequently trial new ideas to improve the welfare of animals in their care.



Finally, I have given talks at the local IAT meeting. This provides a useful forum to discuss our scientific aims in the context of animal welfare. This really provides a great forum to discuss the latest 3R best practice.

Explain the choice of species and the related life stages

The mouse offers a useful system to study the reproductive system. Mammalian germ cells have a distinct life cycle from other vertebrates. Furthermore, mice are a genetic tractability system in which many resources to both study DNA repair and germ cell biology and development exist.

As we are interested in the life cycle of germ cells we will use animals at various stages of the life cycle to capture cells at those different stages.





84. Mechanisms controlling immune cell killing

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

The aim of this project is to understand the molecular mechanisms controlling killer cells of the immune system.

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

Why is it important to undertake this work?

Killer cells of the immune system play a critical role in defending the body against cancer and viral infections. Understanding the mechanisms that control killing by these cells will improve the design of new immunotherapies.

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

We will provide new information on pathways that are required by killer cells of the immune system. We will publish our findings in peer-reviewed, open-access journals. Findings will be presented to the scientific community at conferences and to the wider lay-audience via outreach activities including videos available on the internet.



What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short term the scientific community will benefit from new information about pathways required for killer cells of the immune system. Given the important role that killer cells now play in new immunotherapies the knowledge gained in this study should also improve cancer treatment.

How will you maximise the outputs of your work?

We will disseminate our research output via collaboration with other scientists, scientific publications, presentations at conferences and via public engagement.

Species and numbers of animals expected to be used

- Mice: 3400

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

They will be killed using a schedule 1 procedure. These mice may also be used to generate new genetically altered lines, to cryopreserve these lines and to rederive existing lines into high health animal units.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Genetically modified animals used are not expected to exhibit any adverse effects. Animals are monitored every day and will be immediately killed by a Schedule 1 method if they exhibit 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 species)?

No adverse effects are expected.

What will happen to the animals at the end of the study?

- 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 parallels between mouse and human are well understood and mice are known to provide excellent models of human disease. This allows us to study mutations that cannot be studied in man and generate results that are directly relevant to humans.

What was your strategy for searching for non-animal alternatives?

Human cell lines can sometimes be used as an alternative.

Why were they not suitable?

Very few human cell lines are available to study the function of the immune system.

Reduction

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

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

In the last year we have used just under 600 mice; thus we estimate we will use 3000 mice over the next 5 years.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The use of mice expressing a single T cell receptor (T cell receptor defined mice) to activate killer cells permits the generation of 20 fold more cells per mouse. In this way the overall number of mice required is greatly reduced.

We also include a protocol for embryo derivation so that we can cross gene deleted mice to T cell receptor defined mice. This process means that fewer generations of mice have to be bred to achieve this cross, and hence reduces the number of mice required.

What other measures apart from good experimental design will you use to minimise numbers?

We have optimised culture conditions to generate and maintain killer cells in culture. We have also optimised gene deletion in cells in culture that should reduce the number of gene deletion mice 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use genetically modified mouse strains that do not develop any adverse effects.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice provide the best defined model of a vertebrate immune system. The parallels between mouse and human are well understood and mice provide excellent models for human disease. In this project we only use animals to produce the cells required for experiments that we conduct in dishes in the laboratory. No animals are used in experiments, just their cells and tissues.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We continue to advance the use of improved in vitro cell culture, genetic manipulation and human cell lines as viable alternatives.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The department provides continually updated guidance on experimental refinements. Use of the website from the NC3Rs (<https://www.nc3rs.org.uk>) and LASA (Laboratory Animal Science Association) will also be made.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We constantly review methods for replacement, reduction and refinement provided by the scientific literature and our support team within the facility, attending workshops to stay up to date.

Explain the choice of species and the related life stages

Adult mice are required to generate and preserve new genetically altered lines. Adult mice enable us to generate the greatest number of mature killer cells per mouse, and use fewer mice.





85. Mechanisms of gene and cell regulation by small RNAs in infection and inflammation

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

No answer provided

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

We aim to understand the way that cells in our body communicate to control infection and inflammation and to determine the molecules that they use to do this. We study one specific type of molecule, ribonucleic acid (RNA), which controls gene expression inside of cells and has recently been shown to be transmitted between cells. Our first aim is to identify the genes required for RNA transmission between cells and determine the role of RNA communication in terms of the ability of the immune system to mount a response to, and control, infection. We have also discovered that viruses and parasites produce their own RNAs during infection that can alter host gene expression and can be transmitted between cells. Our second aim is to determine how pathogens transmit their RNA to host cells and the importance of RNA transmission on pathogen survival in order to develop strategies to block this virulence mechanism.

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

What are the potential benefits that will derive from this project?

This work fills in key gaps in our understanding of the molecules that pathogens use to establish chronic infections in animal hosts. This information is needed to advance new drug strategies to treat infection, in the face of mounting resistance of existing therapies.



Our research will also build new understanding on how immune cells communicate with one another using RNA. Immune cell function and signaling are central to inflammatory and infectious diseases as well as cancer and our basic research could inform new disease therapy strategies.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Our studies use mice as a model organism and we expect to use up to approx 15,000 mice over the course of 5 years. These animals have specific gene modifications and the majority will be used in the breeding program to supply postmortem tissues for analysis. Approx 30% of the mice will be used for maintaining the *Heligmosomoides polygyrus* hookworm parasite model which can not be maintained in culture or for studying the immune responses during pathogen infection with maximum moderate severity. Approx 300 rats will be used over the course of 5 years: 200 for maintaining the hookworm model *Nippostrongylus brasiliensis* and 100 for antibody production.

Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The majority of animals are used in the breeding program or as donors of organs that are manipulated *ex vivo* in the lab and have a “subthreshold” severity rating. A proportion of animals will be subject to procedures that are “mild” or “moderate” severity rating and experience only very mild, if any, forms of discomfort.

However we are trying to understand how the immune system controls infection and it is important to understand this with a diverse range of pathogens, some of which induce a degree of pathology. In the case of some bacterial and viral infections there may be moderate severity however we expect all mice to make a full recovery and less than 1% death. It is expected there will be moderate severity due to irradiation of mice in bone marrow chimera experiments however damage is mainly restricted to the hematopoietic and intestinal mucosa and mice are expected to make a full recovery. A small fraction of the immunisation experiments will use CFA which could lead to granuloma formation and ulceration however the incidence is expected to be low (1%) for the duration of the project. The mice will be humanely killed at the end of the experiment.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Our studies require us to examine the ability of the immune system to respond to pathogens and the ability of pathogens to survive in an animal host. The immune response is a highly complex process involving multiple cell types that work together and pathogens have evolved to utilize and control very specific niches inside their hosts. We can not study



these all of these processes without an intact physiological immune system. Where ever possible we use cell culture systems to address specific research questions.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Our experiments are designed carefully to enable us to interpret results and draw conclusions with the minimal number of mice. We build on our previous work and consult statisticians to ensure this is the case. We continuously look for ways to reduce the number of animals used including new tissue culture models that simulate the cell composition and structure of body organs.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The mouse is the most appropriate animal species based on the extensive understanding of the immune system, the tools available to study immune signalling and the similarity of pathogenesis in infection models to those observed in humans. Mouse is the smallest laboratory animal species that can be used to model the interaction between a mammalian host and intestinal parasitic nematode. While the majority of our studies involve mice, a small number requires rats (for parasite infections and generation of antibodies, with minimal harm) as the most appropriate, or only, species for these studies.





86. Mechanisms of Myelination and Ion Channel Distribution

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

To gain an understanding of the development and function of our nervous 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?

The communication between our sense organs, such as our eyes or skin, and our brain and spinal cord occurs through electrical signals that are carried by our nerves. Nerves are composed of bundles of neuronal extensions called axons. Axons conduct these signals through the regulated flux of sodium and potassium ions over the axonal membrane through specific channels. Many of the axons in our nerves are insulated by a fatty layer called myelin, which is made and maintained by support cells called Schwann cells and oligodendrocytes. The myelin layer speeds up nerve signalling enormously, allowing us to respond rapidly and with great accuracy to any physical challenge coming our way. The way myelin speeds up signal conduction is by clustering different sodium and potassium ion channels to high density at regularly spaced regions along the axon.



The importance of this arrangement and density of ion channels becomes apparent when the integrity of the myelin layer is damaged as in multiple sclerosis, Charcot Marie Tooth disease or in diabetes, which alters or even block conductance. But how these ion channels are arranged and kept in specific regions is largely unknown. Understanding how these channels are distributed will aid the development of strategies to harness the nervous systems capacity for repair in demyelinating neurological diseases.

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

In the coming years we hope to gain a deeper understanding of how the insulating myelin sheath around axons in our brain and nerves is made and maintained and how this sheath contributes to the electric properties of the axon. This understanding is important as it opens new avenues for clinical treatment of the many devastating neurological diseases in which the function of the myelin sheath is compromised or even destroyed. We will publish our findings in peer reviewed scientific journals. The project will provide training for graduate students and results will be incorporated into the teaching to students at different levels.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

short/intermediate term:

Development of *in vivo* biotinylation technology will benefit other scientists interested in identifying protein interactions that underpin functional outcomes.

Our GA modified animals are of interest to other scientists and we have distributed our animals to many laboratories, either directly or through the Jackson lab.

Training for postgraduate students.

Longer term:

Generation of new knowledge of Kv1 biology and function. This will benefit researchers and clinicians working on immune mediated encephalopathies involving Kv1 channels.

This research project is of strategic importance and close interactions with other research groups with an interest in myelin and myelin-related diseases ensures that our work contributes directly to the capacity and capability of this larger research community.

Our studies will have a wider resonance as it will impact our understanding of myelin and axonal function in, for example, the hippocampus and cerebellum of the adult and aging nervous system. As such this work is of direct relevance to scientists working on other aspects of neuronal biology.

How will you maximise the outputs of your work?

Dissemination of new knowledge through publication in peer-reviewed open access journals, seminars, conference attendance and poster presentations.

Species and numbers of animals expected to be used

- Mice: 5200



Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The genetic alteration in these mice allow us to study the function of these genes in the development and function of mice by taken out nerve tissue and analyse alterations in its shape and composition. For this the animal will first be culled by an approved method. Additionally, we can study the effect of gene alterations on the behaviour of the animal such as the way it moves, grabs objects and explores it environment.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We don't expect an adverse impact on the general health and behaviour of the animal apart from a number of situations where we model a human clinical disease such altered motor behaviour or seizures. In these cases the animals will be culled well before the impact on the animal becomes severe.

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

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

The vast majority of animals used in this study will not experience any adverse effects during their lifetime. Around 10% of animals will experience mild discomfort as they receive drugs, administered orally or through injection. A small number of animals that model human disease will experience some adverse clinical effects. This is a small number (<5%) and we are actively working to replace these animals with animals that have genes deleted only in a subset of neurons and glia cells.

What will happen to the animals at the end of the study?

- Used in other projects
- Killed

Replacement

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

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

The development, function and response to trauma of the nervous system of vertebrates including mice and man cannot be fully studied in a petri-dish or with computer models. Mice have a sufficiently evolved nervous system to readily compare to humans. Thus



general principle we learn from our animal work will guide work on humans. To learn how the genes we study here affect the function of the nervous system and how it affect posture and movement we will have to this in mice. Cell culture models are intimately integrated in the current programme but still require the breeding of genetically altered mice and the isolation of neurons and Schwann cells from mice and rats (Schwann cells).

What was your strategy for searching for non-animal alternatives?

The development of Schwann cells and neurons from cultured cells is in its infancy and promising results have been achieved, However, in its current state this approach does not provide a viable alternative for animal based research.

Why were they not suitable?

The method is limited in that it is unreliable, takes very long, is very expensive and can only address interactions between glia and neurons. When this method further matures it will contribute to the further refinement and reduction of the use of animals. These alternative methods would not allow us to address the physiological and behavioural role of these genes.

Reduction

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

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

This is based on historic use and power calculations. These studies require a large number of different genetic mutants and most of the mice will be used for breeding to maintain stock

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Experiments are carefully planned and controlled so that only the minimum numbers of genetically altered animals are maintained in the breeding colony. Colony size for each mouse line is routinely discussed and kept at a minimum. When we know the strength of the effect of a genetic alteration we can quantify this and perform calculations to estimate the minimum numbers of animals we need to use in an experiment to obtain reliable results

What other measures apart from good experimental design will you use to minimise numbers?

REDACTED that will not be used within a 12 month period will be terminated. All REDACTED will be preserved by freezing sperm samples so that lines can be revived through in vitro fertilisation at a later date.

We will use male germ line specific Cre recombination to generate null alleles for Adam22, Adam23 and Lgi4 sperm. Homozygous embryos and postnatal animals can be obtained



with a higher frequency (50% versus 25%) from crosses with a heterozygous female, reducing the number of animals 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice have a well-developed nervous system that resembles that of humans in all key aspects studied. The ability to modify the genome of the mouse at will and the availability of functional assays make the mouse the species of choice to model human disease states relevant to the objectives of this programme. We are using, where possible, genetic methods that will only affect a limited group of cells in the nerves and brains so that we limit or even completely eliminate distress in our animals.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We are using embryonic tissues and neonatal animals to address some of our questions but posture and movement of the animal can only be studied in adult animals.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We use specific genetic techniques to minimise welfare costs for the individual animal and have defined clear endpoints to ensure that no animal will experience adverse effects beyond moderate. Animals subjected to experimental procedures will be closely monitored and humane endpoints applied. We will follow good practice suggestions by the nc3Rs, such as the single use of syringes

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

ARRIVE and the Nc3Rs website.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will be notified of advances in the 3Rs through regular email updates from the National Centre for the replacement, reduction and refinement of animals in research and through the Crack It website. Further information is obtained through the scientific literature.

Explain the choice of species and the related life stages



The development and function of the nervous system of vertebrates including mice and man cannot be fully studied in a petri-dish or with computer models. Mice have a sufficiently evolved nervous system to readily compare to humans. Thus, the mouse is ideal to study developmental and functional aspects of the nervous system that is of direct relevance to our understanding of human nervous system development and function in health and disease. We are particularly interested in how the genes under study affect our peripheral nerves and general motor behaviour.





87. Mechanisms regulating local and distal immune responses in barrier site health and inflammation

Project duration

5 years 0 months

Project purpose

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

Key words

immunology, infection, gut, mouth, lung, mucosal, inflammation

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The overall aim of this project is to define critical pathways that control local and long-distance immune responses when we are healthy and when we have inflammation of the places where our bodies meet the outside world (termed barrier surfaces). We will particularly focus on the gut and mouth barrier surfaces.

Currently, the mechanisms by which immune responses are controlled in health and inflammation at the barrier surfaces of the gut and mouth are incompletely understood. We also aim to understand how inflammation of one organ, such as the mouth can lead to inflammation in another organ such as a joint. Better understanding could lead to improved treatment of patients with inflammatory diseases such as inflammatory bowel diseases (IBD), rheumatoid arthritis (RA) and gum disease.

The overall aims of this project, therefore, are to understand the factors that control establishment of immune cell populations at barrier sites, such as the gut and the mouth, in health and, subsequently, how following infection or inflammation they provide protection and eventually healing.

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



What are the potential benefits that will derive from this project?

Using our new-found knowledge of barrier immunity, we hope to inform development of novel therapies to: (i) prevent, or improve outcome in, infectious diseases that target barrier sites such as worm infection; and (ii) treat the plethora of inflammatory diseases that affect barrier sites such as inflammatory bowel diseases (IBD) or periodontitis. These findings are also likely to be relevant to animal health as the mechanisms we define are likely to be ubiquitous between many species.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Mice are the only species to be studied, 7000 will be used in total over a five-year period. We anticipate using approximately 5350 mice for experiments, 800 untreated but genetically altered mice for investigating immune cells isolated from these mice and an additional 500 mice as breeders to generate the mice used on this license.

Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Over half of the experimental animals will undergo infections, or treatments, that will lead to mild generalised discomfort. A typical example of a mild severity experiment would be general discomfort following infection with natural parasites of mice, followed by transient irritation during an injection or immunisation aimed at treating the parasite infection. In some models of human disease used on this license, including colitis, periodontitis and arthritis, the animals may experience discomfort at the site of inflammation. For example, in colitis this would be the large intestine or in periodontitis the gums. Animals in which these types of inflammation will be induced will account for about 30% of mice used on the license. Every effort will be made to limit this discomfort to the shortest time possible and to administer painkillers to alleviate discomfort. In a very small number of animals (<1%), discomfort may become more pronounced in which case the animal will be immediately and humanely killed.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

The body's immune system operates as a co-ordinated response involving multiple cells and molecules at a variety of body sites that at present cannot be accurately modelled solely by using cell culture systems or computational approaches. In particular, the relationships between inflammation in one organ (e.g. the gums) and its effects on another (e.g. the arthritic joint), currently, cannot be reliably established outside of animal models. Where possible we already utilise tissue culture systems to inform our animal experiments.



Over the course of the experiments, knowledge from the animal models will be utilised to better refine new tissue culture approaches to complement and replace animal studies wherever possible.

Reduction

Explain how you will assure the use of minimum numbers of animals.

We will use appropriate statistical expertise to ensure that we design experiments using the minimum numbers of animals required to generate meaningful results. All experiments will be designed to the highest possible standards to ensure reproducibility and limit unappreciated experimental bias. For example, blinding of researchers to treatment groups. We maximise the information gained from each individual animal through the use of the most advanced technologies enabling extensive analysis of small numbers of immune cells.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

In order to understand the mammalian immune system, we use the mouse as a model system as it is the best understood animal in terms of how the immune systems works and has remarkable similarity to other mammals, including humans. The mouse system also provides us with many tools to precisely define immune responses, thus ensuring that the precise mechanisms that underlie inflammation can be well-established. The specific infection and inflammation models used have been carefully selected to ensure that relevant features of human disease processes are recapitulated and that maximal information can be obtained with the minimal harm to the animal. Key general measures of limiting harm to the animal include: (i) daily monitoring of experimental animals using carefully designed criteria to ensure animals are not experiencing unexpected discomfort; (ii) use of painkillers, where possible, to alleviate discomfort; (iii) utilisation of experimental approaches that result in mild generalised discomfort in preference to local inflammation, where information gained is not impacted (e.g. infection with parasites rather than utilising the colitis model); (iv) limiting discomfort to the minimum time necessary to obtain relevant and usable information; and (v) immediate and humane killing of animals experiencing pronounced discomfort.



88. Mechanisms underlying atherosclerosis

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Atherosclerosis is the process by which arteries become progressively narrowed by fatty deposits in their walls. It is the principal cause of coronary heart disease and stroke. Recent evidence suggests that inflammation of the arterial wall is an important contributor to atherosclerosis, but the specific mechanisms underlying this remain unknown. This project will identify the specific inflammatory pathways contributing to atherosclerosis, as well as novel treatments targeting this which may subsequently be tested in the clinic in patients with atherosclerosis.

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

What are the potential benefits that will derive from this project?

This project will increase our understanding by which atherosclerosis develops; and will also provide novel therapeutic targets which may in the future be exploited to decrease the burden of cardiovascular disease in humans.

Species and numbers of animals expected to be used



What types and approximate numbers of animals will you use over the course of this project?

We will be using 9,000 mice, of which 4,000 will be used for breeding and the remaining 5,000 will be used in experimental studies, over a 5 year period.

Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Animals will be fed a high-fat diet, which will cause the development of atherosclerosis in their arteries.

This is necessary, since the project is aimed at elucidating the mechanisms that underlie atherosclerosis and how these mechanisms may be modified to prevent the progression of this condition. It is expected that no animal will suffer harm or welfare issues as a result of high fat feeding.

Some animals will receive drugs in order to assess their effects on atherosclerosis progression. Such drugs may cause adverse effects: for example, anti-platelet drugs such as aspirin will be used, which may predispose to increased bleeding; or antibodies may be used, which can rarely cause allergic reactions. In most cases we expect animals to not feel unwell as a result of receiving such drugs; but in all cases, the animals will be closely monitored for expected side effects of the drugs used, as well as for any more general welfare issues or for any signs of suffering. At the end of an experimental procedure or if it becomes necessary to alleviate suffering at any time then animals will be humanely killed.

Some animals may undergo surgery to remove their sex organs (ovaries or testicles), to examine the effects of sex hormones on atherosclerosis progression. Some animals may undergo surgery to insert slow release pellets of certain drugs or pumps placed under the skin that will slowly release drugs over time. None of these procedures is expected to result in any welfare issues or suffering in themselves. However, with all surgery, animals will experience a little discomfort similar to that experienced by patients postoperatively, but this will be controlled by use of pain relief before, during and after surgery. Again, at the end of an experimental procedure or if it becomes necessary to alleviate suffering at any time then animals will be humanely killed.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

We are interested in understanding the mechanisms that give rise to the physiological changes in the arterial wall during inflammation. Whilst we can study individual cell types in culture, there is currently no lab technique not involving animals that can replicate the physiological changes in the way that atherosclerosis develops in living animals and humans. Where possible we do undertake experiments using human cells in culture.



Reduction

Explain how you will assure the use of minimum numbers of animals.

We are committed to maximising the amount of experimental information we obtain from each individual experimental animal, enabling us to hopefully minimise usage yet at the same time without subjecting any animal to increased associated harms. In some cases, we will perform imaging of animals on a number of occasions during their lifetime, which will enable multiple data sets to be collected in the same animal, and the data thus collected over their lifetime will then be linked to post mortem tissue findings within the individual animal at the end of the study. Such data collected from each animal is more robust scientifically as it comes from the same animal at different time points, and gives a better overall picture from the data perspective than data sets from different animals. Collecting data at multiple time points in the same animal reduces animal use, as the alternative would be to cull a number of different animals at individual time points to collect that data.

We will use good experimental design practice, where the number of animals can be calculated for each experiment (using appropriate statistical techniques and power calculation) . We will use single strains of mouse in our research and use multiple organs from the same mouse in order to keep animal usage down. We will also try to minimise the amount mice we breed in our research.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

We are using mice as they have an immune system comparable in complexity to humans and are the most frequently used model for the human immune system. The apo E knockout mouse in particular is a widely used genetically altered animal model, that is a well validated and accepted model of atherosclerosis. We are mindful at all times to minimise welfare costs to the animals we use. All of our protocols are designed to minimise any welfare costs, and in the event that adverse welfare is unavoidable animals are monitored closely and welfare endpoints are rigorously applied, so that animal suffering is kept to a minimum. For example, it is well established that apo E knockout mice can develop a skin condition known as ulcerative dermatitis, which can result in the animal feeling itchy and damaging their skin through scratching, and we will monitor our mice closely for this and treat promptly and vigorously; but if there is any sign of deterioration or suffering despite such treatment, those animals will be humanely killed without delay.



89. Mechanistic insight into, and assessment of novel therapies for, preeclampsia and fetal growth restriction

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Mice	pregnant, adult, neonate, juvenile, embryo
Rats	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 is the aim of this project?

In rodent models of pregnancy complications to:

improve understanding of the mechanisms underpinning preeclampsia, a maternal syndrome characterised by maternal high blood pressure and worsening kidney function, and fetal growth restriction, the poor growth of a baby, with a specific focus upon the placenta and maternal/fetal blood vessel function.

assess the effectiveness of a number of candidate therapeutics at improving short and long-term outcomes for both the dam and offspring.

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



Why is it important to undertake this work?

Poor function of the placenta can result in a number of complications for mum and baby. These include preeclampsia (PE), a maternal syndrome characterised by high blood pressure and poor kidney function, and fetal growth restriction (FGR), a baby that does not grow as well as it should do. Together, PE and FGR affect around 10% of all pregnancies and increase the risk of stillbirth. Additionally, both PE and FGR place mum and baby respectively at increased risk of heart disease and other adulthood diseases such as diabetes as they get older. Despite the importance of PE and FGR, there are no current therapies to treat them. The major reasons for this are (i) we do not fully understand the reasons why poor placental development and function occurs in some pregnancies and (ii) drug companies are reluctant to invest significant money into designing drugs for use in pregnancy given the additional risks of treating both mum and baby and especially the risks of harming the baby. As such, studies that identify the reasons why some placentas function poorly in pregnancy are key to improving understanding of PE/FGR and to identify new drug targets. There is also a need to test both new and existing drugs/therapies, especially those that we know are safe in pregnancy, in appropriate animal models of PE/FGR to see if they have the potential to be used in women.

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

There will be several expected key outputs following the end of this project. New information on placental and blood vessel function in preeclampsia and fetal growth restriction will be disseminated via research publications and research conferences, both within our immediate field and to more general physiology/scientific audiences. These studies will also generate important preclinical data and identify new candidate treatments for testing in clinical trials. If successful, these approaches may improve the future health of mothers and their babies after a pregnancy complicated by PE or FGR. Our group, together with our obstetric colleagues with whom we work closely alongside, has a proven track- record of taking data obtained in animal models and advancing this knowledge to clinical trials.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short-term, data from this project will likely benefit a number of groups. Scientists and clinicians in the reproductive and physiological fields will benefit directly from knowledge gained in these studies that help to shed light on mechanisms underpinning poor pregnancy outcomes in high-risk groups, such as women with advanced maternal age or women with obesity. Obstetricians caring for these women will also benefit from this knowledge as it will help to inform future clinical care in these groups of patients. Importantly, pregnant women are also likely to benefit from this work, both in terms of improved management of higher-risk pregnancies and in cases where preeclampsia and/or fetal growth restriction has been detected. In the longer-term, towards the end or after the completion of the work outlined in this project, patients and obstetricians are likely to directly benefit from new treatment options for preeclampsia and/or fetal growth restriction.

How will you maximise the outputs of your work?

We will report findings in reputable scientific journals, both within the field of reproduction and in more generalised scientific journals, ensuring maximum scope. We will also



communicate findings at scientific meetings attended by scientists, obstetricians and midwives. We will continually build upon and seek out new collaborators who are able to help us to better understand the reasons why placental function is poor in some pregnancies and not in others, and use this knowledge to identify new treatment options for preeclampsia and fetal growth restriction. When studies do not work as planned or treatments do not improve pregnancy outcomes, we will also report this data as this is also key information for scientists, obstetricians and patients to know. We regularly engage with our patient information panel who help to shape the direction of our research and we will continue to seek their advice and communicate findings to them and also the general public via regular community education and outreach events.

Species and numbers of animals expected to be used

- Mice: 6300
- Rats: 1700

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, animals in this project will either be used to inform us about maternal cardiovascular health (e.g. blood pressure, cardiac function, blood vessel function) and placental function in health and disease (preeclampsia and fetal growth restriction) or to assess the effectiveness of candidate treatments at improving short and long-term outcomes for mum and baby. Animal models used will demonstrate aspects of preeclampsia and/or fetal growth restriction; examples include mice of advanced maternal age, obese mice, mice exposed to a low oxygen environment (to mimic what happens in some cases of fetal growth restriction) and rats infused with substances to simulate what is observed in preeclamptic human pregnancies.

For our obese model, mice will be fed a high fat diet for several weeks before, and during, pregnancy. To study the effects of advanced maternal age, female mice will be aged to approximately 9 months of age before mating. For mice in a low oxygen environment, mice will be housed in their normal cages but also within a chamber which allows us to reduce oxygen levels (for the majority of the duration of pregnancy) similar to what is experienced when living at high altitude. For our rat model of preeclampsia, rats will undergo a short surgical procedure in mid-pregnancy to implant a device under the skin to administer a substance over several days, resulting in increased maternal blood pressure.

In these models, we will assess cardiovascular health (of dams and their offspring) and placental function (e.g. transfer of nutrients from mum to fetus). This may include measuring blood pressure up to three times during the pregnancy, which requires restraint of mice. Additionally, a small subset of animals will undergo anaesthesia to allow us to perform assessments of blood flow to/from the placenta using ultrasound (non-invasive) and a further sub-set of animals will be required to have blood pressure assessed under terminal anaesthesia near the end of pregnancy.



In sub-sets of animals, we will administer treatments we feel have the potential to improve outcomes for mum and infants (e.g. reducing maternal blood pressure, improving fetal growth). The majority of these treatments can be given via drinking water or the diet but in some instances, animals may undergo injections under the skin or into a vein for several consecutive days or undergo a short surgical procedure to implant a device under the skin to administer the drug over several days. As above, we will measure blood pressure up to three times during the pregnancy which requires restraint of the animal and a small subset of animals will undergo anaesthesia to allow us to perform assessments of blood flow to/from the placenta using ultrasound (non-invasive) and a further sub-set of animals will be required to have blood pressure assessed under terminal anaesthesia near the end of pregnancy. In the rat model of preeclampsia, we also wish to monitor long-term health of the previously pregnant animal, including those that will be treated with blood-pressure lowering drugs immediately after the end of pregnancy (via drinking water). This will involve drug treatment via drinking water and assessments of heart and blood vessel function by ultrasound under general anaesthesia. For some animals who are treated during their pregnancy, we will allow the pregnant female to litter down as normal and then perform assessment of blood pressure and early indicators of diabetes (by measuring how well the animal handles a glucose (sugar) challenge) in offspring on up to 2 occasions, a few weeks apart. This will involve restraint of mice in order to measure blood pressure and a single injection into the abdomen (for glucose studies) followed by blood sampling of the tail vein (a single needlestick).

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The animal models chosen in this project will demonstrate aspects of preeclampsia and/or fetal growth restriction. Aside from these clinical signs, we do not expect any of these models to demonstrate serious adverse effects or abnormal behaviour. The majority of treatments will be via drinking water and/or diet and we do not expect any adverse effects of this dosing strategy. When we need to perform injections, these will result in momentary discomfort/pain but no lasting effects. Our surgical procedures are limited to insertion of a device under the skin to deliver substances (e.g. drugs) which will be performed under general anaesthesia. We expect animals to demonstrate normal behaviour within a day of this procedure but may demonstrate mild pain/discomfort which will be controlled via painkillers. When we measure blood pressure in mice, this will involve restraint of the animal. This will provide a brief discomfort but is unlikely to cause long-lasting harm/stress. Assessment of glucose tolerance involves a single injection and needlestick into the tail vein, again providing a brief discomfort but is unlikely to cause long-lasting harm/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 species)?

Most of the animals in this project will reach a threshold of no more than mild severity, as determined by interventions/procedures that cause only a transient, mild discomfort and no long-lasting harm. Where there is a need for repeat procedures e.g. measurement of blood pressure under restraint and an assessment of glucose tolerance, blood flow via ultrasound, this may move to a moderate severity. For animals undergoing general anaesthesia (including more than once), the duration/nature of the recovery may be such that it reaches a moderate severity.



What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

One of the reasons why so little progress has been made in developing drugs for pregnancy diseases is that clinical trials testing treatments in pregnant women are very difficult and ethically challenging. Thus, in order to assess the effectiveness of potential therapeutics for use in pregnancy, the use of animals is a necessary step in the translational pipeline.

What was your strategy for searching for non-animal alternatives?

We always run experiments using human placental tissues and maternal tissue biopsies (e.g. omental tissues derived from caesarean section deliveries) in the laboratory alongside animal experiments as a first step in determining effectiveness of candidate therapies in women. Additionally, by performing studies on placentas/blood vessels from cases of FGR/PE, we can gain insight into vascular and placental function. Computer modelling, particularly for nutrient transport processes, is currently inadequate for our needs.

Why were they not suitable?

Experiments using human placenta cannot inform us of any general beneficial or harmful effects to mother and fetus or their function when a blood supply is intact. Additionally, performing measures of placental function at different points of gestation are highly informative; this is currently not possible in women when studies on placenta are only possible after delivery of the baby/placenta at the end of pregnancy. Computer modelling of the pregnant woman remains some distance from realisation with our present state of knowledge.

Reduction

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

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

For the studies described in this project, we have performed power calculations to inform us of the minimum number of animals required to produce an effect that is biologically/clinically relevant. Experiments are designed to compare disease effects i.e. normal versus complicated (e.g. FGR/PE) pregnancies and/or to assess the effects of



treatment (treatment versus a control group). These calculations are largely based upon data from our own research group (from studies similar to those described herein). Where no previous data is available, we will carry out pilot studies to base future power calculations upon.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

During planning for this project, we have had regular meetings with local statisticians and the experimental plan/power calculations have been performed in agreement with both parties. We have also consulted the NC3R's experimental design assistant to aid with planning and to inform our discussions with statisticians.

What other measures apart from good experimental design will you use to minimise numbers?

We have several years experience of these types of experiments and will utilise this experience to enable efficient breeding regimes. We will keep the number of animals to a minimum by making as many observations/measurements as possible on individual animals (also aided by the fact that each litter comprises multiple pups) and by removing as many tissues as appropriate for later analyses. This ensures that from one pregnant animal, we can obtain multiple datasets. In addition, we will allow other researchers to share tissue from animals used in this study 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The models described in this licence represent well characterised models of PE and/or FGR without significant adverse effects i.e. that do not cause significant pain/suffering or a change in behaviour of the animal but do show the clinical signs we require. When treatments are administered, we will prioritise the diet or drinking water as preferred routes which represent the least invasive methods. Only if this is not possible, will injections or insertion of minipumps underneath the skin be employed.

For invasive procedures (e.g. insertion of minipumps under the skin), anaesthesia will be used alongside careful monitoring of the animals to ensure they are not in discomfort, using painkillers as necessary during and after procedures to minimise pain.

Whilst we do not expect any adverse reactions from our candidate therapeutics, the use of treatments directly targeted to the placenta will potentially further limit any off-target effects.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?



As we require knowledge on placental function a mammalian species is essential for our work. Mice and rats have a uterus and placenta similar to that in women and also allow us to study genetically modified strains, as well as those with dietary/environmental modifications, that have aetiologies or disease symptoms similar to those found in human PE/FGR. Such accurate disease models are not available in less sentient species. The models of PE and FGR described within this licence largely represent established animal models in which to assess placental/vascular function and in which to test candidate therapeutics. In order to assess candidate therapies, we require animals to be treated for several days, generally via the diet/drinking water, that does not cause lasting harm or serious adverse effects. Terminal anaesthesia is not appropriate for the majority of studies described in this licence, but is used when the most information can be gained from an invasive procedure (e.g. placental nutrient transport).

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

When animals are on treatment protocols, we will ensure they are assessed daily by researchers and that all staff involved in husbandry are fully aware of the need for additional monitoring of these animals. For new treatments, we will monitor animals more regularly to ensure there are no unexpected adverse effects. When general anaesthesia and surgery is required (e.g. insertion of minipumps underneath the skin) we will ensure that anaesthesia is at an appropriate depth for the animal to feel no pain and administer painkillers during/after the procedure to ensure any potential harm is minimised. These animals will be closely monitored until they are fully recovered from anaesthesia and thereafter. When restraint is required (e.g. measurement of blood pressure in conscious animals) we will ensure animals are appropriately trained beforehand to become used to the restraint device and thus reduce stress during procedures; this will also lead to less variable data meaning we can use less animals.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will continually remain updated with the latest findings of the NC3R's including the continued use of the experimental design assistant (EDA) and ensure statistical advice is provided throughout the course of the project. When published alternative models or approaches are available which represent an improvement upon current practices, we will perform studies (including pilot studies) to confirm their effectiveness and ensure that all users are fully trained and adopt these approaches if they are deemed an improvement on previous practice.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will regularly consult the NC3R's website and engage fully with the regional programme manager of the NC3R's to ensure we are kept informed of latest developments and opportunities to implement new alternatives/refinements; an example of this is our commitment to the recent drive by the NC3R's to avoid picking the mice up by the base of the tail, even for brief periods, which has been shown to increase levels of anxiety/stress. In addition, we will keep up to date with relevant literature within the field including attendance at research conferences in order to make continued assessments about whether there are further ways to refine our experiments and/or reduce/replace the



animals we use. When alternative models or approaches are available which represent an improvement upon current practices, we will perform studies (including pilot studies) to confirm their effectiveness and ensure that all users are fully trained and adopt these approaches.

Explain the choice of species and the related life stages

Our work focuses upon complications of pregnancy such as preeclampsia, characterised by maternal high blood pressure and deterioration in kidney function, and fetal growth restriction, a baby that does not grow as well as it should. These outcomes increase the risk of poor short and long-term health for both mum and baby. Poor function of the placenta is key to the onset of these complications and so we require models of pregnancy that demonstrate similar placental structure and function to women, in both health and disease, hence the choice of mice and rats. Mice will be primarily used but for some protocols, there will be a necessity to use rats. Justification for the use of rats includes that for some candidate therapies, e.g. kynurenine, the signalling pathways in rat are comparable to human, which is not the case in mice. Additionally, in order to assess postnatal interventions to improve maternal health following a preeclamptic pregnancy, we will use a well characterised and published rat model of preeclampsia which is clinically-relevant and enables us to focus upon a single pathway known to be important in the onset of PE.





90. Mechanistic strategies for tackling chronic liver disease

Project duration

5 years 0 months

Project purpose

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

No answer provided

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 is the aim of this project?

The over-arching aim of this project is to apply insights from human chronic liver disease to discover and develop effective treatments that are tailored to 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?

Liver disease is a silent killer and is on the rise. It is now the biggest cause of death in the UK in those aged between 35-49 years old. If detected early, liver disease can often be reversed, but many people present with advanced liver scarring (fibrosis or cirrhosis) when the options for treatment are limited. There is an urgent unmet clinical need for effective



treatments and preventative strategies for cirrhosis and its complications, to improve outcomes for patients. Increasingly, human liver samples and clinical data can be used to uncover promising targets for treatment, but carefully selected animal models are still required to investigate the effects of new or known medicines for different liver conditions.

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

There is a liver disease crisis in the UK. Since 1970, deaths due to liver disease have increased by 400%. Every day, over 40 people die from liver disease in the UK. Yet there are no medicines 'on the market' to treat liver scarring (fibrosis or cirrhosis) or non-alcoholic fatty liver disease (NAFLD) which is the commonest cause of liver disease affecting 25% of the global population. New treatments are urgently required, as are diagnostic tests to identify people at risk of developing advanced chronic liver disease.

The key outputs from this project will include new information about how liver disease develops, why it progresses in some individuals more than others, and how it could be reversed using novel treatment approaches - this information will be obtained primarily from human data initially (including genetics, analysis of liver biopsies and electronic health records) and used to discover promising new treatments and diagnostic tests that will then be studied in animal models before proceeding to future clinical studies. Given the importance of this work, it is anticipated that other outputs will include: scientific publications, new research collaborations with other academics or with Pharmaceutical and Biotech companies, increased public awareness of liver disease/NAFLD), and potentially patents and products if new medicines, tests or devices are developed.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The main beneficiaries of these outputs will ultimately be patients - through access to new and effective treatments for chronic liver disease. Although it can take time for new medicines to reach patients in the clinic, we have previously shown that it is possible to move promising treatments (such as the molecule relaxin or macrophage cell therapy) from the laboratory into clinical trials in less than 5 years. Another potential benefit is education and public empowerment. Among patients with diseases most associated with NAFLD (type 2 diabetes, obesity, and hypertension) only 6% had heard of NAFLD (Continuum Clinical, 2019). Low public awareness is a barrier to preventative health strategies and to clinical trial enrolment. The work in this project will form part of our broader public engagement strategy.

In a shorter time-frame, the research community will benefit from the project through new insights into liver disease and potential approaches to develop better treatments and diagnostic tests. We will ensure that fellow researchers realise these benefits by making our protocols and data as accessible as possible.

How will you maximise the outputs of your work?

The results of this work will be used to establish new collaborations with other scientists and with Pharmaceutical and Biotech companies to generate further research funding and to speed up the development of new medicines or tests that could benefit patients.

The research findings will be published in high impact open access scientific journals and presented at national and international meetings. We will also engage with the British Liver



Trust and liver support groups, where appropriate, to disseminate the research through their websites and networks. We will craft plain English summaries and visual representations for the public section of our institutional website, and for the annual International NASH Day to ensure our research has global reach. They will also identify opportunities and produce materials for participation in Science Festivals, public lectures and other events. We will use social media judiciously (e.g. Twitter, LinkedIn) and continue to interact with print and broadcast outlets to showcase our work.

Species and numbers of animals expected to be used

Mice: 600

Rats: 300

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, liver disease will be studied in mice or rats using special diets or substances that cause chronic liver damage over 4-12 weeks. Animals may receive treatment with a new therapeutic agent during or after the development of liver disease. Blood samples or imaging tests that are relevant to human health may also be performed to better understand the effects of treatment.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

To replicate the advanced stages of human chronic liver disease and its complications requires models where animals may display clinical signs of liver disease such as jaundice, ascites (fluid in the abdomen) or weight loss. However, we have used these models for many years and have learned how to minimize the chances of unnecessary suffering or harm. Animals are very closely monitored for signs of distress and killed in a humane manner if necessary.

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

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

The mouse and rat models of fatty liver and liver fibrosis that we will use in this project are classified as moderate severity.

What will happen to the animals at the end of the study?

- Killed



Replacement

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

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

Animal models are necessary to study complex biological processes such as liver scarring (fibrosis) and non-alcoholic fatty liver disease (NAFLD) where multiple cell types in a body organ are constantly interacting with each other, and also to capture the circulatory changes associated with chronic liver disease in a manner that reflects the human situation as closely as possible. As yet, laboratory culture dish ('in vitro') experiments simply cannot model this complexity. For this, we require animals - even lower organisms, such as flies, do not have the same level of tissue complexity that we find in mice and human.

Additionally, animal models are critical to demonstrate proof that new drugs have a good chance of working in humans and to obtain information about the likely dose required and any potential safety issues.

Wherever possible, we are planning to incorporate non-animal methods to achieve our project aims.

What was your strategy for searching for non-animal alternatives?

We are using a range of non-animal methods and only using animal models for specific experiments, such as studying the effects of a new treatment or the utility of a new diagnostic test in the liver and other body organs.

We will use computers to undertake initial screening for new drugs ('in silico' studies), in vitro experiments that mimic simple processes that occur in a living animal (such as fat accumulation in liver cells), and human liver samples that we have already collected. During the course of the project, we also aim to establish a new technique using small slices of human liver which are kept alive in an incubator for several days - this might prove very useful in studying the effects of new drugs.

Why were they not suitable?

Non-animal techniques are suitable for many aspects of the work in the project, but cannot yet be used reliably to fully understand and measure the effects of a new treatment or a new diagnostic method (such as MRI).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 group has many years of experience in the design and conduct of rodent liver disease models. We have used data from our previous published studies to estimate the minimum number of animals needed to show a true effect from a treatment whilst at the same time maintaining sufficient numbers for the experiment to be meaningful. We use biomedical statisticians to advise on our study designs and analysis methods when required.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We used the NC3R's Experimental Design Assistant to map out experimental studies. In certain circumstances non-invasive techniques (such as magnetic resonance imaging) can enable us to track disease in an individual animal over time and will help to reduce the numbers of animals required.

What other measures apart from good experimental design will you use to minimise numbers?

We use the very best models available that mean each animal gives us essential information, with little wastage. Tissues from the animals we use in research are stored and kept for future analysis, meaning that we do not need to repeat the experiments to generate further tissue.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use models of liver fibrosis and non-alcoholic fatty liver disease (NAFLD) in mice and rats - they are very well-established and provide a reliable system to understand how liver disease is caused and to determine the effectiveness of new treatments. It is important to use models that reflect specific types of liver disease in humans (e.g. the carbon tetrachloride model most closely mimics human liver injury due to alcohol or toxins, whereas dietary models exhibit key features of human fatty liver disease). Therefore, the observations we make in these species can be directly translated to the human condition. The clinical complications of cirrhosis only occur at the more advanced end of the disease spectrum. Therefore, in certain studies where we are interested in investigating or treating cirrhosis, models of at least moderate severity are often required. We will minimize any potential suffering by using the least amount of liver injury required for each model, the earliest possible study endpoints, and judicious administration of anaesthesia and analgesia.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Rodent models are extremely well-characterized and validated for studying the progression and regression of liver fibrosis and for evaluating the efficacy of new



therapeutics. Lower organisms, such as flies, do not have the same level of tissue complexity that we find in mice and human and are not suitable for this type of research.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

A major component of our research programme is the development of non-invasive techniques (imaging, blood tests) which may refine our approach – for example, by allowing experimental models to be stopped sooner if these tests give an earlier indication of response to a drug.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will adhere to the ARRIVE guidelines and regularly consult the NC3Rs website to ensure best practice.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I follow the NC3Rs on LinkedIn and Twitter. My group will also regularly consult the NC3Rs website and attend our local annual 3Rs symposium.

Explain the choice of species and the related life stages

Once potential new treatments for chronic liver disease are identified we need assess their effects in animal models of liver disease that mimic, as close as possible, the human condition before they can be evaluated in clinical trials. Models of liver disease in adult mice and rats are most commonly used for this purpose and several drugs are now in advanced clinical development based upon supportive data accrued from rodent studies such as those proposed in this project.

Animal models are necessary to study complex biological processes such as liver fibrosis and its complications in a clinically meaningful fashion. As yet, laboratory culture dish ('in vitro') experiments simply cannot model this complexity. Furthermore, animal models are critical to demonstrate proof that new drugs have a good chance of working in humans and to obtain information about the likely dose required and any safety issues.





91. Medicine Palatability Assessment

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

To identify any aversive taste in new medicines during their development or once they are marketed.

To develop an internal library of the various ingredients of medicines including the active ingredient, the medicine itself, to understand how they affect the taste of a medicine or predict how a new medicine may taste.

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

Why is it important to undertake this work?



Palatability of medicines is important. If medicines are not palatable this can stop people taking them and the medicine may not be effective. This is the case for medicines taken orally, inhaled and intranasally, and is a particular issue for children.

Work performed under a previous project licence provided an invaluable way to identify and help mitigate taste aversion before administration to humans. Prior to this, aversive taste associated with potential new medicines was a risk and could not be measured until early clinical trials. An aversive taste at this stage can be sufficient to stop a medicine going on the market (a rare event) or require modifications to the formulation of the medicine. To address these issues, the medicines palatability test was introduced to REDACTED to predict any taste issues and minimise changes to medicine formulations as they are developed and tested.

There is evidence of genetic and cultural components to taste, and REDACTED takes due account of the likely ethnicity of their target patient population, especially children. The close correlation between the rat brief access taste aversion (BATA) test and human data is evidence of the translatability of the BATA to humans.

With REDACTED's focus on reducing the failure rate of medicines to successfully treat patients for unmet clinical need, the BATA is used across therapeutic areas as a key and unique tool in medicine development.

This test is predominantly used in the early stages of discovering a new medicine or in the first stages of clinical trials.

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

A reduction in the number of substances that fail or experience delays late on in development due to taste related issues.

Inclusion of palatability data in documents provided to the regulatory authorities during the approval process for new medicines.

External presentation/ publication of refinements to the BATA test.
Publication of academic/ industrial collaborations.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Output from the BATA will contribute to key medicines reaching the patient population in a timelier manner and reduced taste issues should ensure more people take their medicine, particularly children.

Early identification of palatability issues (particularly for inhaled medicines) could prevent more animals being used to test toxicity and humans going through clinical studies to develop medicines with little hope of the medicine getting to market by deselecting these compounds at an earlier stage in development.

Inclusion of pre-clinical palatability data in regulatory documents enables REDACTED to inform formulation development plans, particularly for medicines intended for children. Improved formulations with optimal taste qualities will help ensure patients take their medicine and reduce the issues caregivers typically experience when administering aversive tasting medicines to patients.



Increasing information in our database will provide REDACTED information on the effect of ingredients of a medicine as well as the active ingredient, the medicine itself.

Information that is not intellectual property of REDACTED will be shared, including further validation and refinements to the BATA test. This will help towards a standardised research approach, improving reproducibility and reducing repetition of animal studies.

How will you maximise the outputs of your work?

Collaboration with academic, industrial and non-government organisation partners, and agreeing development strategies will maximise alignment and minimise unnecessary repetition. Outputs will be disseminated via presentation at conferences, external meetings and scientific publications.

Species and numbers of animals expected to be used

- Rats: 300

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Rats will be water restricted for a typical 21 hours (up to 22 hours) per day for a maximum of 4 days. Rats will be placed into a chamber (attached to the lickometer) with restricted space and exposed to a series of test formulations for up to 30 minutes per day and for up to 4 consecutive days. The rats choose whether or not to drink each of these test formulations. Following each 30 minute session, rats are returned to their home cage and provided with drinking water for typically 2.5 hours (minimum of 1.5 hours) prior to the next water restriction period.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Transient body weight loss due to the water restriction period of up to 8%. Under the previous project licence, no animals experienced 10% weight loss at any time during studies and all animals showed weight gain with a maximum weight loss of 4% within the rehydration period.

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

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

Mild (100%)

What will happen to the animals at the end of the study?



- Killed

Replacement

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

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

Without the use of animals, accurate prediction of palatability can only be determined during clinical development of medicines. To get to this point several safety assessment studies, usually in rats and dogs, must be conducted for regulatory authorities.

There are 25 bitter receptors currently identified in humans and there is a complex interrelationship between these receptors, and between receptors which detect bitterness and those involved in the other taste senses which is difficult to model in non-animal alternatives. No fully validated non-animal alternative for the assessment of palatability currently exists.

Rats possess taste receptors similar to humans and the BATA has shown excellent ranking of test compounds when they are compared. Because of the tests sensitivity (slight changes in palatability affect the rate of licking) we are able to obtain high quality data, improving the accuracy of clinical prediction. The complex interrelationship between receptors in man also exists in the rat and the BATA can be used to assess the taste masking effect of medicine formulations. Thus, at present, the rat BATA is the best model of the clinical situation.

What was your strategy for searching for non-animal alternatives?

We have previously worked with an academic group to develop an electronic sensor array system (e- Tongue) which can mimic biological taste.

We have worked with another academic group to determine the potential utility of the amoeba *Dictyostelium discoideum*, a unicellular organism sometimes referred to as 'slime mould', as a model of measuring bitterness.

We are currently working with an academic group using machine learning to develop a computer algorithm to predict bitterness based on a substances chemical structure.

We are aware of work that has been conducted using cells transfected with bitter taste receptors and human taste cells that can now be cultured *invitro*. These technologies were (and continue to be) reviewed as part of a cross-industry UK working group of which our Company is part of.

Why were they not suitable?

Data from the e-Tongue has not been sufficiently reliable in 'blinded' assessments for us currently to use it with confidence it will provide accurate data.

Limited data generated in the *Dictyostelium discoideum* model suggests reasonable correlation with human data and might be considered as a triage model (prior to the rat BATA) in due course. However, further validation is required before a decision can be



made regarding its utility. In addition, *D. discoideum* do not respond to non-bitter taste sensations (sweet, sour, salt, umami) and do not have the complex interrelationship between receptors seen in rats and human, so it could not be considered as a full replacement for animals.

Machine learning computer algorithms are currently not capable of determining the intensity of bitterness to an acceptable level of accuracy. However, this technology is developing rapidly and may be a potential triage model (prior to the rat BATA) in due course.

The use of taste cells *in vitro* to assess the taste properties of substances is complex and requires more research. At present, these types of cell-culture assay are not capable of modelling the complex interrelationship between receptors and do not provide an accurate picture of overall taste liability of substances.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 anticipate running one study every two weeks (excluding holiday periods) for the five year duration of this licence. This equates to 24 studies per year for five years, using 12 rats per study. Assuming each rat is used an average of five times (4 re-uses) and accounting for variation in demand, we estimate a potential use of 300 rats.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Statistical advice will be sought to optimise study design and minimise animal usage.

Information collated in a database on medicines and their ingredients, and their effect on palatability will be used to advise on medicine development, reducing the need to assess these in the rat BATA and ideally avoiding further animal use in the first place. In addition, fewer substances with such liabilities will be used in other animal studies under the authority of other project licences.

Prior to committing to animal studies, potential test substances will be assessed for solubility and chemical stability. Substances with very low solubility and hence only limited interaction with taste receptors will be deemed to be of 'low taste risk' by default and rat studies will not typically be performed. Substances which cannot remain chemically stable for the duration of a study session (approximately three hours) will not typically be assessed under this licence. Under exceptional circumstances (for example assessment of a formulation for children prior to inclusion in regulatory documents), substances with limited chemical stability (between one and three hours) which are stable enough for use on individual lickometer sessions may be assessed under this licence.

Batches of animals which have undergone one BATA study (of one week with suitable periods to access water in between individual tests) have been shown to be suitable for re-



use in subsequent sessions (where there are sufficient substances needing to be tested or validation studies needing to be performed). The advantage of re-use is that one fewer water restriction, and one fewer habituation session in the lickometer would be needed per study compared with using a naïve batch of animals. Welfare assessments indicate limited impact to the animals of 23h (reduced to 21h in most cases) water restriction with periods to access water once testing is complete.

What other measures apart from good experimental design will you use to minimise numbers?

Use of prior knowledge captured in our database will enable more informed study design and avoid the unnecessary use of substances which themselves might cause taste aversion.

Noise levels will be kept to a minimum during studies to ensure rats remain focused on the task at hand and are not distracted, for instance by radios, unnecessary talking or other sudden noises. This will reduce variability of the data generated and thus the number of animals needed by avoiding having to repeat studies or use more animals to minimise variability.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

There is a large amount of literature supporting the use of rodents in assessments of palatability, indicating the physiology, molecular biology, and behavioural aspects of taste in rodents are similar to human.

Evidence generated during the course of previous BATA studies following single and multiple uses suggests rats experience no signs of acute or chronic stress, normal behaviours, growth rates, blood chemistry and haematology, with no abnormal post mortem findings. Body weight loss (typically below 10%) does occur during the water restriction phases, but this is transient and recovered once animals have access to water again.

Rats will be maintained in 'tower' style caging with various in cage levels/ platforms and an enriched environment that improves the rate of learning and habituation to novel tasks and thus reduce the variability of the data generated. Rats are housed in standard caging where a specific scientific or welfare reason has been identified.

Welfare assessments will be conducted that include body weight monitoring; skin 'tenting' as evidence of sufficient water intake, body condition and general observational assessments.



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Most non-mammalian species (e.g. zebra fish) are not considered to be suitable alternatives due to their different mechanisms for detecting taste to mammals.

Although taste cells develop during the embryonic stage of mammalian development, the complex interrelationship between receptor types cannot be modelled in non-mammals.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

During the course of previous licences, various refinements have been assessed and we will continue to explore opportunities for further refinement. Previous assessments have included:

Reduction of the typical water restriction phase to 21 hours (from 23 hours)

Reduction of the number of times rats are offered substances to taste (from 3 to 2) per session, reducing the typical lickometer session to 30 mins (from 45 mins)

Use of the home cage for post session access to water (replacing use of a separate chamber)

Use of enriched 'tower' caging to improve the rate of learning to novel tasks

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The published principles and philosophies behind the PREPARE (2018) and original ARRIVE (2010) guidelines have been incorporated into the sponsoring company's internal project planning standards of care and standard operating procedures. All work carried out under authority of this licence will undergo assessment of the study design during planning stages as part of a peer review process that is based on those guidelines and will include statistical consultation. Facilities and processes are audited by independent bodies such as AAALAC International which has published guidelines and procedures to ensure work is carried out to high ethical and humane standards. The following published documents will advise on experimental design, animal welfare and husbandry during the life cycle of this licence:

Kilkenny C et al (2010). Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. PLoS Biol 8(6).

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

Percie du Sert N et al. The ARRIVE guidelines 2019: updated guidelines for reporting animal research. BioRxiv. 2019: 703181.

NC3R's - Responsibility in the use of animals in bioscience research: expectations of the major research council and charitable funding bodies (2019).



Guidance on the operation of the Animals (Scientific Procedures) Act 1986. (Home Office 2014).

LASA - Guiding principles on good practice for animal welfare and ethical review bodies. (2015)

Prescott MJ, Lidster K. Improving the quality of science through better animal welfare: the NC3Rs strategy. *Lab Animal* 46(4):152-156, (2017).

Review of harm-benefit analysis in the use of animals in research. Report of the Animals in Science Committee Harm-Benefit Analysis Sub-Group chaired by Professor Gail Davies (Nov 2017).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The role of REDACTED's Named Information Officer (NIO) includes the sharing of animal welfare, best practice and 3R's related information. The NIO also liaises directly with REDACTED project licence holder network through their meetings, and also raises this type of information and discussion points at the Animal Welfare and Ethical Review Body (AWERB). The licence holder for this work regularly attends both these groups and is also aware of the 3R's related work of the 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). Experience to date has shown that 3R's issues and advances are highlighted, discussed and actions are implemented within REDACTED centrally and effectively via these forums. In addition, the licence holder regularly liaises with scientists specialising in laboratory animal behaviour and with a special interest in rodent taste models, and attends/ presents at relevant scientific symposia.

Explain the choice of species and the related life stages

Data from the rat is more predictive of human responses than non-mammalian or non-animal alternatives. Rats and humans possess similar taste receptors and respond to the majority of substances (particularly bitter) in a way that is highly translatable between the rat and man. Use of the rat as a model for predicting taste aversion in humans has been validated in rats between 8 weeks and approximately 1 year of age within which the rat response has shown to be acceptably consistent. Published studies suggest rats outside of this age range give a more varied and less translatable response to bitter tastes.





92. Melanoma formation, immune responses and evaluating novel therapeutic approaches and agents

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

To learn more about how melanoma (an aggressive form of skin cancer) arises, how it interacts with the immune system and to exploit this knowledge to develop new therapeutics

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

Why is it important to undertake this work?

There are approximately 16,000 new diagnoses each year in the UK of melanoma, resulting in approximately 2,300 deaths per year. For most patients diagnosed with advanced melanoma (melanoma that has spread to other anatomical sites) there is little prospect of a cure and survival time can be a few years or even only months from diagnosis. However, through acquiring knowledge of the molecular, cellular and metabolic processes that go wrong during the formation of melanoma it has been possible to develop



drugs and cellular therapeutics that significantly delay disease progression in many, or occasionally even cure patients. The number of these therapeutics are limited, however, and treatment resistance is still a major hurdle. More research is needed in order to expand the pipeline and understand and deal with the issue of resistance.

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

By the end of the project we hope to have validated several novel potential therapeutic targets for treating advanced melanoma (which may be of relevance to other malignancies). We also aim to generate understanding of how melanoma cells develop resistance to immune checkpoint inhibitors, drugs that boost T cell responses [T cells being part of the immune system]. Potentially, resistance mechanisms may be reversed using other drugs. Further, we aim to generate and evaluate the safety and efficacy of a novel chimeric antigen receptor (CAR). CARs are assembled from multiple proteins and introduced into T cells (ultimately from a cancer patient). They guide T cells to a cancer surface protein and then activate the T cell so it can destroy the cancer cell. Our findings will be disseminated at scientific meetings and in publications and our reagents licensed where appropriate for commercial development.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Our research is guided by our desire to benefit melanoma patients. Within the next five years, we anticipate that other scientists in academia and pharma may build on the knowledge we generate and disseminate to develop novel treatments and diagnostic tools. However, new therapies resulting from our discoveries may take several years before they reach patients, as drugs need to be identified and rigorously tested before they can be prescribed.

How will you maximise the outputs of your work?

We will maximise the output of our work by collaborating and by publishing at the earliest opportunity through pre-print servers and then Open Access journals. Further, we will disseminate our findings at international meetings attended by academics, clinicians and industry representatives. Currently we collaborate with a Biotech company and are thereby able to transfer our knowledge directly to an organisation capable of developing cellular therapeutics and testing them clinically.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



Some animals will only be used for breeding. However, in the majority of animals, we will establish melanoma tumours at superficial sites. We will track how the tumours are developing over several weeks to months, sometimes using medical imaging approaches. Certain animals will be dosed with therapies, including drugs and cells. We will then monitor closely over days to weeks how well the therapy is working and whether it is causing any side effects.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Cancer growth within an animal, as in a human, can cause detrimental effects. We monitor weight and specific behaviours in the animals that would indicate pain or distress and have well designed endpoints to minimise any suffering. There is a risk that we may observe toxicity with treatments. To limit this, we use therapies at doses that have previously been shown to be well tolerated using the route of least severity and the minimum number of doses to produce an anticipated anti-tumour effect. If the monitoring described above indicates toxicity, we reduce therapy dose and/or stop treatment.

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

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

Minimal if any side-effects are anticipated in animals used for breeding. All procedures used for animals with tumours are moderate. We will not allow tumours to grow bigger than 1.5 cm³, nor to interfere with vital functions. At the end of experiments animals will then be humanely killed and tissues may be taken for further laboratory tests.

What will happen to the animals at the end of the study?

- 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 multiple cell types implicated in the process of cancer formation and progression and treatment response are all present only in an animal host. Additionally, in the case of drugs or cell therapies, we are intending to address whether the therapeutic will be absorbed, metabolised and distributed effectively to have its effect without causing significant toxicity. Ultimately this requires an intact, living organism (notwithstanding the caveat that mouse and humans have important pharmacological differences).

What was your strategy for searching for non-animal alternatives?

Human tumours and tissue culture were considered as non-animal alternatives and will be used extensively before animal experiments.



Why were they not suitable?

Non-animal alternatives can not be used to test all our hypotheses due either to our inability to manipulate the system (as with human tumours, outside the context of a clinical trial with its own attendant ethical issues) or as with tissue culture because we cannot yet replicate all the important features of the in vivo environment and certainly not holistically. Co-culture systems can begin to address interactions between different cell types a couple at a time but not the entire spectrum of interactions. In particular, immune responses require the orchestrated activities of multiple cell types located at distinct anatomical sites, with various cells needing to migrate from one anatomical site to another.

Reduction

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

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

For individual experiments, pilot and historical data are used in power analysis to predict optimal group sizes. Numbers are then scaled to encompass the anticipated/desired number of experimental scenarios, incorporating controls, different doses and independent replication.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Experiments will be appropriately controlled and mice of the same age, genetic background and source used to reduce the variability of results and to produce highly consistent data. An on-site statistician has advised us on design and power analysis, alongside use of the NC3Rs Experimental Design Assistant. Bias will be avoided by random allocation of animals to treatment groups. Where possible and practicable, endpoints will be assessed by a technician who is unaware of the treatment the animal has received. Experiments will be designed carefully to avoid other sources of bias such as week-to-week or operator-to-operator.

What other measures apart from good experimental design will you use to minimise numbers?

The use of real-time data monitoring of cells (e.g. luciferase mediated imaging of tumour cell development) has considerably reduced the group sizes required for significant experimental 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.



Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The mouse is the lowest vertebrate that offers an in vivo situation (anatomy, physiology, metabolism) relevant to human cancer and that can be manipulated in a manner that will generate data relevant for the treatment of human cancer. As such, the mouse is the most appropriate in vivo model to achieve the stated objectives.

Sampling blood, the injections (subcutaneous or tail vein) to introduce tumour cells or drugging by gavage, intraperitoneal or intravenous injection are routine procedures and will be performed by highly trained staff, which ensures brevity of the procedure and the lowest level of discomfort. Needles will only be used once when sampling or administering substances to mice. By responsibly considering the adverse effects associated with the regulated procedures, mechanisms are in place to minimise these (e.g. appropriate analgesic regimes for pain relief).

To reduce any suffering of tumour bearing animals, animals will be humanely killed as soon as tumour formation is sufficient to yield satisfactory data and always before animals become moribund, manifest severe pain or lose significant weight (which is closely followed).

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Cancers develop in vertebrates. We model melanoma both in zebrafish and mouse. Mouse is more similar to humans than zebrafish and this is critical both for using reagents like drugs developed for human targets and for translating findings back to the clinic. Cancers develop over many weeks to months, so use of terminally anaesthetised animals or immature animals is not practicable. Also immature mice lack a functional immune system which is important for our research.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Immune-based therapies may be associated with toxicity related to the underlying mechanism of anti-tumour activity. Local experience reveals that this has most frequently been associated with weight loss in less than 20% of all experimental animals and has been managed through close observation and the provision of mashed food and nutritional gels rather than hard pellet. Wherever possible, animals will be provided with analgesia as detailed in the relevant Protocol to control adverse symptoms associated with the treatment. In all cases, judgement will be used to assess whether the duration of such symptoms is excessive or whether the animals appear stable and / or show an improvement in symptoms over time suggesting that these effects were transient.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

All tumour studies will be conducted in accordance with the guidelines for working in rodent models of cancer as described by Workman et al (2010). (Guidelines for the welfare and use of animals in cancer research. Workman P, Aboagye EO, Balkwill F, Balmain A,



Bruder G, Chaplin DJ, Double JA, Everitt J, Farningham DA, Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR, Eccles SA. Br J Cancer. 2010 May 25;102(11):1555-77).

LASA guidelines will be followed regarding volume of blood samples, and drug dose volume limits.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

By reading 3Rs literature and participating in 3Rs workshops locally and nationally. Through discussing refinements with my NACWO, NVS and HO inspectors.

Explain the choice of species and the related life stages

Adult mice will be used as

The mouse immune system has been intensively investigated and is the most advanced in terms of understanding.

The necessary reagents are available and usually well tested which is not the case for most other species.

The mouse represents the lowest level of sentience that most accurately reflects the human system for immunotherapeutic studies

Correlative studies between mouse and man indicate the potential for results to translate between the species.

The immune system is mature in adult mice and only they are big enough to host tumours, are more practical to treat, and more able to tolerate any side-effects compared to younger mice.





93. Metabolism and Residues of Agrochemicals in Food Producing Animals

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

No answer provided

Animal types	Life stages
Cattle	juvenile, adult, pregnant
Goats	juvenile, adult, pregnant
Pigs	juvenile, adult
Chicken	juvenile, adult

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The intention of work covered by this licence is to determine how agrochemical materials are absorbed by the body of food-producing animals, how they may be changed by the animals (metabolised), how they are broken down and excreted, and what residues remain. The work is designed to meet the requirements of government regulators in Europe and elsewhere, who must agree to the sale and use of these materials in society.

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

Why is it important to undertake this work?



Agrochemicals; primarily insecticides, herbicides, fungicides and other materials designed to kill crop pests, are essential for the prevention and treatment of diseases that commonly occur in our food crops. However after use, a residue of the material may remain in the food stuff, and be eaten by animals. It is therefore important to determine that these materials are not toxic to the animals, and that any parts or products of the animal (eggs, milk and meat) are then safe for humans to eat. Without these studies, it is currently not legally possible to manufacture and sell these materials in the UK, in Europe, or into other markets in the world. Development and use of safer and better materials requires conduct of these studies.

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

Data collected will be information on the absorption of various agrochemical materials by food-producing animals, how the body changes and excretes these materials, and what residues may remain in products which contribute to human food (milk, eggs, meat). The data will be collected to the standards required by government regulators in the UK, Europe and elsewhere, who will make decisions on whether these materials can be safely marketed and used in society.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Our clients, typically commercial agrochemical companies, will benefit from the provision of high quality data. This will help them in their work to produce safer and more effective materials which can be safely made and used without increasing health risks for those who could potentially be exposed to them through the eating of animal-based food.

Enabling development of successful agrochemical materials will benefit society, for example by enabling improved crop yields in multiple regions of the world, which are exposed to a range of biological challenges.

How will you maximise the outputs of your work?

By conducting the work to the expected quality standard (Good Laboratory Practice), the outputs of the work should be readily accepted in all markets of the world.

Collaborations and information exchange with others within the organisation and with our clients, helps to identify and spread information on successful and unsuccessful approaches, and on product development.

Species and numbers of animals expected to be used

- Cattle: 30
- Goats: 60
- Pigs: 10
- Sheep: 0
- Domestic fowl: 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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will be given a chemical material by methods which reflect the expected route that they would be exposed to them. Samples are collected, including milk from goats and cattle (standard farming practices are used) or eggs from hens. Sampling may also include blood samples. Urine and faeces, and the excreta of birds are commonly collected, which requires housing the animals singly, although next to each other, in a small cage which allows the urine and faeces to fall through a grid, typically for about up to two weeks. To ensure that urine is not lost confinement of goats and cattle is typically in a type of stall which prevents them from turning round.

Animals will be humanely killed after the collection period, and tissues may be taken from the animals post mortem, and analysed.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The process of dosing animals or taking blood samples may cause minimal discomfort during conduct; no lasting effects expected.

The materials are not normally expected to cause any significant harm for the animals.

Confinement for collection of excretions may cause a degree of discomfort; a degree of reduced food consumption and/or weight loss may be noted, but this is not expected to be significant based on experience of studies conducted over many years.

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

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

Although no significant harms are normally identified clinically, the single housed confinement of animals for more than a week, particularly if it includes restricted movement, results in a consideration that this is of moderate severity. This is likely to be the case for the majority of animals used in the project, because of the need to collect the excretions of individuals for a sufficient time to complete the scientific aims.

What will happen to the animals at the end of the study?

- Killed
- Kept alive
- Rehomed
- Used in other projects

Replacement



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

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

While non-animal methods are used in some aspects of the programme of safety assessment of new materials, they are currently not able to predict effects on whole body systems or to provide information on how much of a material is absorbed, or how animals change and excrete materials. Crucially, animal products which contribute to food (meat, milk, eggs) need to be assessed for presence of chemical residues which could cause harm to people.

The protocols described in this project are conducted according to internationally-agreed guidelines, and are expected to be performed before government authorities will authorise the marketing of new agrochemical materials.

What was your strategy for searching for non-animal alternatives?

The organisation conducts non-animal tests as part of the programme of safety assessment of new agrochemical materials, but as noted, above, it is still considered essential by scientists and government regulators, to also do work using animals, which this project describes.

Studies of absorption, change or excretion after dosing by mouth still can not currently be achieved adequately without using animals.

Why were they not suitable?

These studies of absorption, change, excretion and residues in materials which may be in human food, still can not currently be achieved adequately without using animals; and so the materials can not be approved for use in society.

Reduction

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

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

The estimates are based on analysis of use of animals in an existing licence authorising work for the same purpose, combined with anticipated upcoming studies.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The animal numbers for studies required by government regulators typically follow those identified in internationally-accepted guidelines, as expected to provide sufficiently significant outcomes.



Some studies use only one animal per study, where the scientific aim can be achieved with this design. In studies requiring multiple animals, previous experience can be used to agree a study design including fewer animals than is advised in the relevant guideline.

What other measures apart from good experimental design will you use to minimise numbers?

A careful process is followed, to understand the specific needs for any particular material for which testing is requested, to understand the purpose of the testing, where in the world a material might be made, transported or used, and what tests may have been already performed/submitted to government regulators.

All studies are performed according to the principles of Good Laboratory Practice (GLP), which is an international standard for the quality of experimental study conduct with animals, and is overseen by an independent agency of the government. Following this practice should ensure the quality of studies and acceptance of results by government regulators worldwide, and also reduce the potential for error.

By fully understanding the needs for testing in each case, and by following internationally-agreed guidelines and GLP, the same study should be acceptable to government regulators in all parts of the world where it is needed, removing any need to do the similar work more than once, and use more animals, to meet the needs of all markets where agrochemical materials may be used.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Materials are given to the animals by the same way in which they would be exposed in the environment, typically by mouth, through the eating of animal feeds).

The dosing methods used are all very well established and common for the animals to be used, and known to cause minimal discomfort based on extensive experience at the site. Similarly, sampling methods follow published guidance where relevant, and animals are kept confined for the minimum time to meet the scientific needs.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The species used are selected based on known standards of outcome which will answer the scientific questions. They are species proposed as appropriate for the work by internationally-agreed guidelines, for the studies which are expected to be performed before government authorities will authorise the marketing of new agrochemicals. The outcomes are assessed over a time period which makes continued anaesthesia impractical, and would interfere with the outcome in most circumstances.



What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Refinement of procedures is an on-going consideration at the site, and new opportunities are particularly assessed if any concerns are identified during or after studies; for example new methods or additional assessments may be included in future study designs as a result of a review. Single housing and confinement in a reduced space are commonly needed in these studies, and ways to improve (enrich) the stall or cage used discussed.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Where required, blood volume limits are within those proposed in the 2001 publication of Diehl et al: A good practice guide to the administration of substances and removal of blood, including routes and volumes.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Both our clients and our colleagues working in the same type of work in other countries, are collaborators who can bring ideas as to how to improve how to conduct our animal studies. Various staff at the establishment have been involved with working groups of the UK National Centre for the 3Rs (NC3Rs), over many years. Staff at the site routinely review published papers in the scientific press, some of which propose refined approaches to conduct of work.

Explain the choice of species and the related life stages

The animals and life stages used in the project are examples of species which contribute to human food. They are also species included in relevant guidance accepted by government regulators in the UK, Europe and elsewhere.





94. Metabolism of Chemicals

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

No answer provided

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 is the aim of this project?

To evaluate how various types of chemical materials are absorbed by the body of animals, how they may be changed by the animals (metabolised), and how they are broken down and excreted. The work is designed to meet the requirements of government regulators in Europe and elsewhere, who must agree to the sale and use of chemical materials in society.

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

Why is it important to undertake this work?

The work is required to help protect the safety of people who are involved with the manufacture and transport of various types of materials, as well as those who may be exposed to them during deliberate use or accidental exposure. Without these studies, it is



currently not legally possible to manufacture and sell these materials in the UK, in Europe, or into other markets in the world. Development and use of safer and better materials requires conduct of these studies.

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

Data collected will be information on the absorption of various chemical materials, as well as how the body changes and excretes these materials. The data will be collected to the standards required by government regulators in the UK, Europe and elsewhere, who will make decisions on whether these materials can be safely marketed and used in society.

Improved methods of conduct of specific data collection processes may be developed during the course of the project.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Our clients, typically commercial chemical companies, will benefit from the provision of high quality data. This will help them in their work to produce safer and more effective materials which can be safely made and used without increasing health risks for those who make, use or transport them.

Enabling development of successful chemical materials will benefit society, for example by enabling improved crop yields, reduced danger to workers, or provision of chemicals as ingredients in safer or better medicines.

The wider scientific community may benefit from publication of refined approaches to animal use.

How will you maximise the outputs of your work?

Our organisation has colleagues who also have experience of such work in different parts of the world. Collaborations and information exchange with others within the organisation, helps to identify and spread information on successful and unsuccessful approaches.

Collaboration with clients (knowledge gained on products).

On-going collaborations with NC3Rs on various aspects of regulatory safety studies, over many years.

Presenting outputs at scientific conferences and contributing to publications in the scientific literature where relevant.

Species and numbers of animals expected to be used

- Mice: 1200
- Rats: 5000

Predicted harms



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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will normally be given a chemical material by mouth or by intravenous injection, and most commonly on a single occasion only. Samples such as blood samples will be taken to assess how much of the material is absorbed after swallowing, and/or samples of urine and faeces to see how much of the material is excreted in various ways. Collecting urine and faeces requires keeping the animals, normally singly, in a small cage which allows the urine and faeces to fall through a grid, typically for about a week, but for a maximum of 14 days.

Some animals will have surgery conducted under anaesthesia, and with use of pain relief, to allow collection of bile from animals after they have been dosed and single housed as described above.

Animals will be humanely killed after the collection period, and tissues may be taken from the animals post mortem (after death), and analysed.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The process of dosing animals, taking samples and confining for collection of urine/faeces can cause a degree of discomfort during conduct. The materials are not normally expected to cause any significant harm for the animals. Surgery can cause some discomfort, but this is prevented or minimised by use of appropriate anaesthetics drugs and pain relief.

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

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

The harms described above are expected to fall about half within the mild category and about half within moderate. Where surgery is performed, as described, this would be noted as moderate severity, and may involve about a tenth to a quarter of the total number of animals. If animals are single housed for a period of days for collection of urine/faeces, that is also considered as a moderate harm. Severe outcomes are not anticipated; if seen in individual animals, these would be reported to Home Office.

What will happen to the animals at the end of the study?

- 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?

While non-animal methods are used in some aspects of the programme of safety assessment of new materials, they are currently not able to predict effects on whole body systems or to provide information on how much of a material is absorbed. It is not currently possible to acquire all of the information on how the body absorbs, changes and excretes materials, without using animals. This information is essential, to protect people involved in the manufacture, transport or use of chemical materials. The protocols described in this project are conducted according to internationally-agreed guidelines, and are expected to be performed before government authorities will authorise the marketing of new chemical materials.

What was your strategy for searching for non-animal alternatives?

The organisation conducts non-animal tests as part of the programme of safety assessment of new chemical materials, eg in vitro metabolism studies are used within programs to aid with translation to human safety and also to confirm suitability of the rodent species with respect to metabolite profiles.

However as noted, above, it is still considered essential by scientists and government regulators, to also do work using animals, which this project describes.

Studies of absorption, change or excretion after dosing by mouth still can not currently be achieved adequately without using animals. Studies of absorption through the skin can be conducted either in animals or by non-animal methods. Generally the non-animal method is suitable in this area of work, using isolated human or animal skin samples.

Why were they not suitable?

There are limits to non-animal skin absorption studies; the skin samples are only usable for up to about 24 hours, so where absorption has to be followed over a longer time period, an animal study will then normally be required.

In some cases, the results of the non-animal method then require use of an animal method to confirm

Also, where skin penetration is shown to be high in the non-animal method, the relatively increased risk for humans will then normally require that an animal study is also conducted, to provide additional information regarding safety for people who may be exposed to the material.

In rare cases, enough is known about the properties of a test item to judge that a non-animal study alone will not meet the scientific need to fully assess human safety. In such cases, an animal study and a non-animal study may be planned to be conducted together. However such a plan must be specifically approved by the Home Office.

Reduction

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



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

The estimates are based on analysis of use of animals in an existing licence authorising work for the same purpose, combined with anticipated upcoming studies.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The animal numbers for studies required by government regulators typically follow those identified in internationally-accepted guidelines, as expected to provide sufficiently significant outcomes.

What other measures apart from good experimental design will you use to minimise numbers?

A careful process is followed, to understand the specific needs for any particular material for which testing is requested, to understand the purpose of the testing, where in the world a material might be made, transported or used, and what tests may have been already performed/submitted to government regulators.

All studies are performed according to the principles of Good Laboratory Practice (GLP), which is an international standard for the quality of experimental study conduct with animals, and is overseen by an independent agency of the government. Following this practice should ensure the quality of studies and acceptance of results by government regulators worldwide, and also reduce the potential for error.

By fully understanding the needs for testing in each case, and by following internationally-agreed guidelines and GLP, the same study should be acceptable to government regulators in all parts of the world where it is needed, removing any need to do the similar work more than once, and use more animals, to meet the needs of all markets where a chemical material may be made, transported or 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Materials are given to the animals by the same way in which people would be exposed during manufacture, transport or use of the materials, most commonly by mouth; occasionally by putting it on the skin, and then comparing how much is absorbed with dosing directly into the blood. In a small number of cases, surgery is performed, under anaesthesia and with pain relief, to allow samples to be taken from the animals.



The methods used are all very well established and common for the animals to be used. The amount of material given is in line with published guidance on minimising discomfort, and/or is known to cause minimal discomfort based on extensive experience at the site.

Similarly, blood sampling follows published guidance on suitable volumes which can be taken while minimising harms to animals.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The species used are selected based on known standards of outcome which will answer the scientific questions. They are species proposed as appropriate for the work by internationally-agreed guidelines, for the studies which are expected to be performed before government authorities will authorise the marketing of new chemical materials.

The outcomes are assessed over a time period which makes continued anaesthesia impractical, and would interfere with the outcome in most circumstances.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Refinement of procedures is an on-going consideration at the site, and new opportunities are particularly assessed if any concerns are identified during or after studies; for example new methods or additional assessments may be included in future study designs as a result of a review. Habituation of animals to restraint (if needed) is a routine process, and the schedule can be amended in response to outcomes for individual animals.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Dose volume and blood volume limits agreed with the animal welfare and ethical review body are based on the 2001 publication of Diehl et al: A good practice guide to the administration of substances and removal of blood, including routes and volumes.

Welfare end-points are developed in general line with publications on the topic, including the NC3Rs document from 2010 on dose level selection for regulatory toxicology studies.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Both our clients and our colleagues working in the same type of work in other countries, are collaborators who can bring ideas as to how to improve how to conduct our animal studies. Various staff at the establishment have been involved with working groups of the UK National Centre for the 3Rs (NC3Rs), over many years. Staff at the site routinely review published papers in the scientific press, some of which propose refined approaches to conduct of work.

Explain the choice of species and the related life stages

The animals used in the project have been shown to provide important information for assessing safety of chemical materials, and this is reflected by the recommendation of the



particular animal species in internationally-agreed guidelines for how to best produce the information required.



95. Mitigating Impacts of River Engineering on Fish

Project duration

5 years 0 months

Project purpose

None selected

Key words

Dams, hydropower, acoustics, telemetry, electricity

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

The project aims to: (1) quantify the impact of river infrastructure (e.g. hydroelectric turbine intakes and pumping stations) on fish; and 2) develop environmental impact mitigation technology, such as behavioural deterrents (e.g. using acoustic/electric fields), to protect fish.

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

What are the potential benefits that will derive from this project?

Information obtained will provide evidence of negative impact of man-made river structures on fish distribution, movement, and survival to help develop and prioritise conservation actions. This will help regulators ascertain whether current methods to protect fish (e.g. fish ladders and screens) are effective for the species of conservation concern, and highlight areas where improvements can be made. Identification and quantification of secondary impacts associated with river structures and their operation, such as the sounds created, will be achieved so that alternative protective measures can be developed.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Fish.



The total number depends on the number of sites selected by the Environment Agency for monitoring. Total maximum number = 22,100 over 5 years.

Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The adverse effect of this study will be related to the marking of fish (e.g. use of elastomer tags, fin clips, and PIT tags) which will require minor procedures involving a hypodermic syringe, tagging of fish which will involve surgery and suturing, and stress associated with exposing fish to sound and electric fields. These will include minor discomfort and elevated stress during the use of anaesthetic and marking /tagging procedure, associated with insertion of hypodermic needles (e.g. to implant visual markers) and surgery associated with implanting a transmitter into the body cavity of the fish. When encountering electric fields likely adverse effects are likely to be minor and relate to those common for sedation and anaesthesia, primarily that motion and breathing is reduced accompanied by a partial loss of equilibrium, but remaining reactive to touch stimuli. The likely/expected levels of severity will range from mild (all protocols except tagging studies involving surgery) to moderate (use of surgery to tag fish). Risk of mortality will be low (less than 1:1000) and any fish exhibiting stress or suffering as indicated by abnormal behaviours and increased rates of gill ventilation as a result of the procedure will be humanely killed with a lethal dose of anaesthetic. In most cases fish will be returned to the wild after completing the procedures.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

It is currently impossible to model movement behaviour of many species of fish in response to conditions encountered at river structures. Live animals must be used to obtain the information necessary to facilitate greater understanding of this so that models may eventually be developed. This project will provide information needed to develop deeper understanding of fish behaviour on which conservation efforts can be based. The approach proposed will use the minimal amount of animals to obtain the information necessary.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Statistical tests will be used to estimate the numbers of fish that should be used to obtain valid results. Through a process of continuous re-evaluation and adaptation of the research methodology, the numbers of fish required will be constantly refined by updating the estimates the number of fish needed while still achieving scientifically valid results. Statistical tests will be used on data collected to inform estimates of numbers used in



subsequent phases of the research. This way the number of fish required to ensure meaningful conclusions will be minimized.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

It is essential that the animals used are the same species for which this research is intended to benefit by developing the most appropriate management strategies based on real world observations. The species selected represent those that of high conservation and/ or economic value as defined by national and international legislation, yet maintain populations that are relatively stable and healthy in the study rivers selected. The selected species also provide representatives of fish with a range of different body morphologies, swimming capabilities, behaviours, and life history traits. All surgical techniques will use well developed and widely used protocols to minimize handling and associated stress. Effects of the techniques will be monitored to reduce probability of causing pain and suffering during future phases. Behavioural traits will be monitored to indicate humane endpoints i.e. the earliest indicator in an animal experiment of severe pain, distress, suffering, or impeding death. Post-surgery behaviour will be closely monitored over the period of recovery to assess deviation from the presurgery condition. The data will be regularly reviewed to assess whether behavioural measures can be refined to enhance the efficiency of identification of humane endpoints based on a relationship between exhibition of aberrant behaviour and resulting deterioration in condition.





96. Modeling liver disease in rats

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

No answer provided

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

When the liver is damaged by fat or alcohol or viruses' scars can form: if the injury is stopped then the scars are slowly degraded and the damage is repaired. However, when the liver is exposed to repeated injury then the scars persist, a process known as fibrosis. In the diseased and fibrotic liver there is an increased risk for cancer to form and grow.

Currently there are no medications which prevent or limit fibrosis and for cancer, the only drug treatment, Sorafenib, only extends life expectancy by ~3 months and often has side effects.

This project has 3 aims;

The first is to understand how scars form in the liver when it is damaged and then to identify new targets for drug development. We then want to test medicines, which have the potential to prevent or reverse scar formation in animal models of liver disease.

The second is to understand how cancer develops in the diseased liver and then test drugs that might prevent or limit cancer development.

The third is to determine how protection from developing fibrosis when the liver is damaged can be passed on from either grandfathers or fathers to their offspring.

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



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

What are the potential benefits that will derive from this project?

The primary outcomes of our work are;

Identify new drugs, which limit scar formation and prevent fibrosis or liver cancer.

Identify biomarkers of disease (e.g. develop a test that will allow us to measure proteins or DNA in the blood that are released from the damaged liver) or use imaging (a simple scan) to help assess how advanced the fibrosis or cancer. Our aim is that the biomarkers and imaging methods will be developed to assess disease in both rodents (to minimise animal use and procedures) but also to be developed as a test that can ultimately be used in patients with liver disease or cancer. For example, biomarkers may include molecules or proteins released into the blood that can be detected by a simple blood test, or an imaging method could be developed as a scan that measures the amount of fibrosis in the liver or the size of a tumour. By performing repeated scans this will tell us if a drug is working and reduces the amount of scar tissue or size of a tumour in the liver.

Clinical Importance:

The number of people with liver is continuing to increase year on year in the UK, primarily due to the increase in obesity, viral infection and alcohol.

Currently, in the UK, more than 16,000 people die in from liver disease, ranking as the fifth most common cause of death. Whilst in the US it is the second leading cause of mortality amongst all digestive diseases. At present, there are no effective drugs to treat liver fibrosis. The only treatment option for patients with advanced liver disease/liver failure is a liver transplant. However, donor organs are extremely limited and in 2009 only 58% of patients on the waiting list received a transplant.

Liver cancer, typically develops on the background of chronic liver disease, effects over half a million people each year, and has a poor 5 year survival of only 10%. The number of people diagnosed with liver cancer is rising at an alarming rate and is now reported to be the 5th most common cancer in men and the 7th most common cancer in women. The poor survival rates are primarily due to late diagnosis and a lack of effective drugs.

There is an urgent need to identify new drug targets and test potential new therapies to treat chronic liver disease (CLD) and liver cancer. Our program of work will help understand how CLD and liver cancer develop and allow us to identify and test new medicines to treat CLD.

This program of work will also hope to develop imaging methods to help assess liver disease/cancer in living animals and also aid the development of blood tests which can predict the stage of disease.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Rats, we have estimated using up to 3500 rats over a 5 year project.



Predicted harms

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

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

All procedures with the exception of protocol 1, which are non-recovery are classified as moderate.

We isolate different cells from the liver and grow them in a petri dish to perform early drug testing and understand how these cells behave under different conditions to help identify new drug targets. Under protocol 1, rats are placed under anaesthesia (into a deep sleep) and the liver is exposed and perfused with solutions that aid cell isolation. This method is a non-recovery procedure.

To cause liver injury in rats we will use either liver damaging chemicals (carbon tetrachloride), surgical models (blocking of the bile duct) or by feeding of western lifestyle diets which are high fat and sugar diets.

The rat will develop liver disease and very rarely show signs of sickness e.g. hunched posture. Very occasionally jaundice (skin becomes a yellow colour) or swelling of the belly may develop in the bile duct surgical model.

For our studies investigating how protection from scar formation in the liver is passed from generation to generation we will also ask if other organs are affected. We will promote disease in other solid organs including; 1. The kidney by surgically tying the ureter, to prevent urine flowing from the kidney to bladder. 2, The lung by putting chemicals into the lung to cause damage. 3. The skin of rats by punching two holes in the skin and watching the wounds heal. In these models, scars will form in the effected organ.

We will administer chemicals to rats to injure the liver and induce liver cancer. The rat will develop liver disease and cancer slowly. The animals may lose weight but tumours will not be allowed to develop to a point where significant clinical symptoms occur.

We do not expect therapies to cause adverse effects, typically the therapies are drugs, which have been used in animals to treat other diseases, but are likely to have value to treat liver disease or cancer. If a compound had not been used in animals before, we would perform small pilot studies to determine drug bioavailability and tolerance. Starting doses would be informed by literature regarding the compound solubility and activity in a test tube and from experiments performed in cells or tissue slices.

We will use imaging machines (e.g. x-ray, MRI or a machine that measures light) to scan the rats with CLD to help assess the level of the disease in living animals. To see the scar tissue we will give special “tracers” which bind the scar or scar cells. The tracers light up or glow, vibrate or release when stimulated and can be seen by the special imaging machines. From our current experience we do not anticipate any adverse effects of imaging.

Replacement



State why you need to use animals and why you cannot use non-animal alternatives.

Liver disease and cancer are complex processes; they develop over many weeks and require lots of different cells both within the organ and within the blood to talk to each other

In our “inheritance” study we are asking how protection from developing fibrosis can be passed from father to son. To achieve this we need to promote fibrosis in the dad, then breed him with a female rat and then injure their offspring and then compare the amount of scarring to rats bred from injured or un- injured parents.

For these reasons these programs of work can only be conducted in animals and not alternative systems such as growing cells or thin slices of an organ in a petri dish.

Wherever possible, we use human tissue to isolate cells or make slices or stable cell line cultures generated from human cells, which we can culture in a petri dish to replace animal models of liver disease.

We also use stored frozen and wax embedded liver tissue collected from previous studies to help answer our research questions and minimize further use of animals.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Studies are always planned very carefully and use statistical analysis to calculate the minimum numbers of animals needed to achieve meaningful results and achieve our research aims.

Primary cells, cell lines and slices of liver tissue are also used to help reduce numbers of animals used. Before testing drugs in rat models of liver disease we first show that they have a “therapeutic effect” in cultured cells and tissue slices, this way we only test drug that we expect/predict to have a positive response.

Using non-invasive imaging is another strategy we employ to reduce group sizes or numbers of time points.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

All of the liver disease models chosen are the lowest severity model that can be used to answer our research questions.

Whilst, the majority of work is performed in mice under a separate project licence, some procedures are necessary to perform in rats because of the nature of the drug or imaging agent being tested or help advance our understanding of the disease biology.

For example, the chemical model (carbon tetrachloride) is very predictable therefore we can predict the disease stage at any given time point. We have lots of experience running drug studies in this chemical model, therefore we know exactly what time to give drugs



and for the length of time to treat the animals. In this model rats receive bi-weekly injections of the chemical into their belly, however before we start the model the rats under a period of handling to minimise the stress of restraint and an injection.

We have good surgical techniques and operating theatres/equipment to minimise the risk of infection. The bile duct ligation model is a surgical model. In this model rats receive pain relief and a high level of post-operative care including soaked diet, a warm environment and fluids as required to minimize stress and suffering. We work with the vets and animal behaviour/welfare scientists to develop clinical scoring system to help assess the animal's well-being and level of disease.

Animals are checked regularly and supportive care is readily provided to minimise suffering and improve the rat's welfare.





97. Modelling, measuring, and modulating the neurobiology of addiction-like behaviour

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Animal types	Life stages
Mice	adult
Rats	adult, juvenile

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Individual variation and susceptibility to developing addiction-like behaviour, as well as the success of treatment strategies, will be compared in (1) animals that vary in motivation, (2) animals exposed to certain or gambling-like uncertain rewards, and (3) animals that experience early-life stress.

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

Why is it important to undertake this work?

Studying the neurobiology of motivation and attention in animals may help us to understand disorders such as addiction people. The proposed research stems from our



finding that certain rats, due to both biology and experience, are highly-attracted to reward-related stimuli may also display addiction-like behaviours. The brain's dopamine system also functions differently in these animals, and this may result from differential expression of specific dopamine-related proteins. Genetic differences in these proteins have been proposed to relate not only to addiction in people, but also ADHD and autism. Our goal is to investigate how variation in dopamine-related neurobiology in animals impacts addiction-like behaviour. Accordingly, we aim to develop strategies for decreasing potential development of addiction in susceptible individuals. Psychiatrists and clinical psychologists treating patients for mental health disorders will also benefit from our results. For example, patients with substance use disorder may be attracted to environmental cues that are predictive of a drug, and these cues may, therefore, promote drug-seeking. Our findings may suggest that it is possible to reduce the motivational power of these cues, either through cognitive-behavioural or pharmaceutical approaches. Beyond these initial goals, the research has relevance to increasing our understanding of the neurobiology of the brain's dopamine system. Thus, individuals who study a variety of disorders will benefit from the results. Following publication, results will be shared in an online data depository. We will encourage secondary analysis of the data.

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

This research will produce novel data which will be published in high-impact peer-reviewed journals, shared at conferences, and presented during invited seminars. Following publication, results (in the form of easily-accessible spreadsheets) will be shared in an online data depository. We will encourage secondary and meta analyses of the data.

Increasing understanding of (within 1-3 years):

the neurobiological and behavioural-cognitive processes underlying individual variation in cue- evoked behaviour and addiction.

how presynaptic regulation of dopamine transmission impacts postsynaptic signalling in dendritic spines and resulting cell body activation.

brain systems that encode habitual versus motivated behaviours, and how these brain systems may become dysregulated in addiction.

the shared neurobiology between drug and behavioural addictions, and the interaction with social experience during adolescence and adulthood.

the influence of drug-taking environment on drug choice

Developing personalised pharmacotherapeutic and psychosocial methods for decreasing (within 2-5 years):

acute and long-lasting motivational states evoked by exposure to reward-associated cues that drive aberrant reward-pursuit.

habitual or goal-directed behaviours involved in reward-seeking or reward-taking.

continued search for reward that occurs even when it is not available.

motivation to pursue reward even when costs of doing so are high.



choice of drug or pursuit of gambling by giving access to social reward.

This research will encourage the continued development of highly translational animal models of human behaviour, thereby refining research techniques (within 1-5 years).

A long-term consequence of researching individual variation in behaviour may be to reduce the total number of animals used, as neurobehavioural differences across rats will be acknowledged and studied with appropriate statistical methods (within 5 years).

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

This research will interest academics who study (within 3+ years):

how neurobiology influences patterns of behaviour evoked by environmental stimuli.
how neurobiology regulates individual variation in motivated and conditioned behaviour.
how neurobiology regulates individual variation in attention and impulsivity.
neurological disorders involving the dopamine system. Such disorders include, but are not limited to, substance use disorders, eating disorders, ADHD, autism, schizophrenia, and Parkinson's disease.

This research will interest psychiatrists, clinical psychologists, and pharmaceutical industries who wish to treat patients for mental health disorders, including those who combine such methods as (5+ years):

cognitive therapies designed to reduce the 'motivational magnet' properties of reward-predictive stimuli (especially those that influence addictive behaviours).

contingency management therapies that offer social rewards

pharmaceutical treatments for decreasing acute states of desire evoked by reward-predictive stimuli.

individualised treatment methodologies for those identified as displaying high degrees of 'sign-tracking' or 'goal-tracking' behaviour.

How will you maximise the outputs of your work?

This research will produce novel data which will be published in high-impact peer-reviewed journals, shared at conferences, and presented during invited seminars. Following publication, results (in the form of easily-accessible spreadsheets) will be shared in an online data depository. We will encourage secondary and meta analyses of the data.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 6000

Predicted harms



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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Some animals will perform tasks in chambers and consume food rewards (up to 6 hours per day, for several weeks). At certain points, animals may receive injections of drugs or control substances to alter their reward-related memories or motivational states (the number of injections an animal might receive will be limited). At the end of this experiment, rats will be humanely killed under anaesthesia. Their brains will be removed, and the tissues will be processed to investigate individual variability in neuronal structure and biochemistry.

Some animals will also undergo behavioural testing and receive, or self-administer, infusions of a drug (up to 6 hours per day, for several weeks). Surgery will be required for animals that self-administer a drug. In addition, some animals may also receive surgeries that enable (a) the injection of pharmaceutical compounds or viral vectors, or (b) the insertion of electrodes, directly into specific brain regions.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Some animals may undergo surgery that will enable the measurement/modulation of brain activity and/or allow them to self-administer drugs directly into the blood supply. Following recovery from surgery, animals will not show clinical signs of distress during behavioural testing.

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

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

A significant portion of animals (approximately 1000 rats and 1000 mice) will experience only behavioural testing and will likely not encounter forms of distress. It is possible animals may be fed a specific amount of food at a certain time of day; this will help to motivate animals to pursue a food reward during behavioural tests. The weight of animals will be carefully monitored for abnormal gains or losses. We don't anticipate any adverse effects of these studies.

Some animals will require surgery; example surgeries include (a) the insertion of a jugular vein catheter to support self-administration of drug (approximately 1000 rats), (b) the implantation of cannula into the brain to enable measurement or modulation of neuronal processes (approximately 2000 rats and 2000 mice), or (c) both of these types of surgery during a single session of anaesthesia (approximately 2000 rats). Surgical procedures are considered moderate in severity. Operations will be performed in anaesthetised animals using standard aseptic techniques, with the use of pain-relief medications. Animals will be carefully monitored for signs of distress multiple times per day for at least one week following surgery. We do not anticipate any long-term adverse effects of these procedures. At the end of this experiment, animals will be humanely killed.



What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 analyse the relationship between brain activity and behaviour, intact behaving animals are required. These experiments cannot ethically (or practically) be performed in people (e.g. implanting intracerebral cannulae).

The proposed experiments will enhance our understanding of the neurobiology of addiction and why some individuals develop addiction, while others do not. Accordingly, we will begin to develop personalised treatments for addiction.

What was your strategy for searching for non-animal alternatives?

Several *ex vivo* techniques will be used, including analysing the striatal neuroanatomy and biochemistry of rats previously monitored for behaviour.

Why were they not suitable?

Since the studies link brain function and behaviour, it is necessary to sample brain regions taken from whole organisms and not from cultured cells. Because the relationship between brain neuroplasticity and behaviour is largely unknown, it is not currently possible to model *in silico*.

Reduction

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

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

Appropriate statistics have been used to minimise the number of animals used. The number of animals used (and experimental design) are also based on evidence from previous studies of individual variation in behaviour.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Procedures have been optimised in our previous research. Where possible, a within-subject experimental design will be used, reducing animal numbers. Based on previous research screening 1000s of rats for Pavlovian conditioned approach, we expect a roughly



equal distribution of behavioural phenotypes. Neuroanatomy or neurochemistry can be assessed in each rat by making separate observations in each hemisphere, thereby reducing the number of animals needed. Rats will be assigned to groups according to a randomised Latin Square Design and maximum effort will be made to ensure that the experimenter will be blinded.

What other measures apart from good experimental design will you use to minimise numbers?

Pilot studies using small numbers of animals will be performed to optimise testing procedures before more extensive experiments take place.

We will record all factors that may impact individual variation in behaviour. For example, in the past, we have found that some rats that are ordered from specific facilities belonging to certain commercial suppliers are more likely to display particular behavioural traits (e.g., sign-tracking vs goal-tracking). Therefore, it is critical to consider what commercial supplier should be used when ordering animals for a given experiment; this will help reduce the number of animals used.

Other researchers at the establishment may perform similar behavioural experiments to those included in this project license (e.g., Pavlovian Conditioning). It is possible, therefore, that brain tissues taken from animals tested under other project licenses at the establishment can also be tested for neurochemical process related to learning, memory, and motivation. Some brain samples from animals used on our other licensed work can be used for addressing the research objectives of the present application (as well as serve as samples for pilot studies). Such samples have been taken into account and have reduced the number of animals required for use in this project license.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

In the proposed experiments, studies in which animals do not undergo surgery (approximately 1000 rats and 1000 mice) are mild in severity and will not result in substantial pain, suffering, distress or lasting harm (PSDLH).

Studies that require animals to undergo surgery (approximately 5000 rats and 2000 mice) are considered moderate in severity. Animals recover quickly from surgery and will not have long-lasting PSDLH. From an experimental perspective, animals mustn't be tested if they exhibit signs of PSDLH; the presence of PSDLH would influence behaviour and thus confound the experimental results.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?



The use of rats and mice enable the investigation of individual variation in complex behaviours and the easy/replicable analysis of brain-region-specific neuronal processes. Less sentient animals (than rats or mice) would not be able to perform the behaviours we are studying, and thus such animals cannot be used.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Every effort will be made to refine procedures and minimise potential sources of distress. Animals will be given suitable bedding, shelter, chew-blocks, and nesting-material in appropriately-sized cages. Health will be monitored at least daily. For injections, safe volume sizes and needle gauges will be used. Experiments will have well-defined endpoints (after specific behavioural observations). Animals will be humanely killed.

All surgeries will be performed aseptically using combined general and local anaesthesia, according to well-established protocols. Following surgery, animals will be closely monitored for at least one week, given post-operative analgesic and anti-inflammatory agents based on Home Office practices and veterinary advice, and provided with palatable food to minimise distress. Individuals performing surgeries will be trained and supervised.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

When designing experiments and sharing our results, we will follow the recommendations of the ARRIVE Guidelines (Animal Research: Reporting of In Vivo Experiments) and the Experimental Design Assistant (EDA) provided by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs).

We will follow the Animals (Scientific Procedures) Act 1986 (ASPA) and the Home Office's Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will regularly undergo reviews of our experimental and surgical procedures with our NACWO and NVS. We will also attend seminars on best practices in animal research that are held at the establishment and conferences we attend. We will follow events, updated documents, and blog/news posts that are highlighted on the website (and in email newsletters) published by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs).

Explain the choice of species and the related life stages

To analyse the relationship between brain activity and behaviour, intact behaving animals are required. These experiments cannot ethically (or practically) be performed in people (e.g. implanting intracerebral cannulae). Therefore, the use of rats and mice enable the investigation of individual variation in complex behaviours and the easy/replicable analysis of brain-region-specific neuronal processes. The proposed experiments build upon our previous research.



Only rats will be used for studies in which animals are trained to self-administer drugs. There are several reasons for using rats, rather than mice, for self-administration, including (but not limited to) more straightforward surgery due to larger body size and better comparison to previous research (e.g., PubMed scientific literature search: "rat cocaine self-administration", ~2,750 articles; "mouse cocaine self-administration", ~430 articles). Also, rats have larger brains than mice; this allows for greater precision in the study of certain sub-regions of the brain (e.g., the core and shell subdivisions of the nucleus accumbens).

Genetically modified mice may be used for the study of specific cell types in the brain. These mice, which are engineered to express certain identifiable markers in specific neurons, are available both commercially and from our collaborators. Equivalent genetically modified rats are not readily available and may be more challenging to engineer.

The majority of animals used will be considered adults. A subset of experiments may use juvenile rats; these experiments will model how, in people, social experiences early in life can influence both the development and persistence of addiction.





98. Models of haematopoiesis, therapy and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

No answer provided

Animal types	Life stages
Mice	adult, 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 is the aim of this project?

This project aims firstly to improve our current understanding of cause and effect relative to each molecular lesion in Primary Immunodeficiency Diseases and metabolic disorders / Lysosomal Storage Diseases with the aim of improving existing treatment and developing new therapies.

Secondly we wish to demonstrate pre-clinical efficacy and safety of cellular and gene therapy protocols and conditioning regimens for treatment of PIDs, LSDs and malignancies, with an aim to improve upon the current vectors and protocols used in existing clinical trials.

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

Why is it important to undertake this work?



Primary immunodeficiency diseases are genetic disorders classically characterised by impaired host defence and an increased susceptibility to infection. PIDs are associated with congenital defects in haematopoiesis and a dysregulated immune function. Due to rapid progress in diagnostic techniques, especially genome sequencing, more than 350 distinct disorders of In-born errors of immunity have been identified, with several new disorders identified every year. Most of the PID patients suffer from recurrent infections, have a poor quality of life, and will die if left untreated. The current treatments for PIDs are 1) expensive e.g. PEG-ADA (a current enzyme based therapy) costs between £200,000 and £400,000 per year compared to a one off treatment with gene therapy. and 2) cause adverse effects such as long-term toxicity.

The expected benefits of this project are firstly improved understanding of primary immunodeficiency diseases and metabolic diseases. Human samples are rare and often in small quantities due to the affected individuals mainly being children. The use of animal models allows an ethical and practical solution to this. Secondly we aim to improve the treatments available for Primary immunodeficiency diseases through the continued development of both cellular and gene therapies. We have shown a clear benefit from previous work where we have moved gene therapy treatment from the bench to the bedside, with the use of murine models crucial to this. We have also increased our understanding of haematopoietic stem cells and our ability to manipulate them. We are continually developing novel vector elements to improve the expression levels and location thus increasing the efficacy and safety. We expect further clinical trials to be started within the lifetime of this licence. Advances in vector development have shown clinical efficacy without the side effects previously seen. The next 5-10 years will see the treatment of these diseases using gene editing approaches, and also based upon vector improvements and increased understanding of the molecular basis of the disease.

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

Outputs are pre-clinical data that will be used to translate novel cell and gene therapy protocols into the clinic and publications in peer reviewed journals of new information for the benefit of the wider scientific community.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The main beneficiaries of this research are paediatric patients affected by monogenic disorders or cancer and their families.

The knowledge accumulated with these studies will also shed light on the properties of different sub- populations of cells involved in haematopoietic system and immune system, such as haematopoietic stem cells or T cells which will be beneficial to the entire scientific community.

How will you maximise the outputs of your work?

The outputs of this work will be used to secure further funding needed for translation of the pre-clinical data into the clinic.



The data generated will be shared with the scientific community through peer reviewed publications and presentations at relevant scientific meetings, including unsuccessful approaches, in line with the principles of 3Rs.

Species and numbers of animals expected to be used

- Mice: 15,600

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will be bred, tagged, marked and genotyped as appropriate. They may be used only for harvest of organs to study a particular disease phenotype at a particular stage of their life or may be used in other experiments.

Animals may be used in transplantation studies after conditioning by Chemotherapy, irradiation or antibody based reagents. Transplantation may be carried out by intravenous, subcutaneous, intraperitoneal, intrafemoral routes in adults, or intrahepatic or supra-facial veins in neonates, or in-utero laparotomy for transplantation of embryos. The engraftment of the transplanted donor cells will be tested by peripheral blood sampling or bone marrow biopsies of the recipient mice. The effectiveness of the immune reconstitution may be tested by challenging with immunomodulators. Animals may be then sacrificed by a schedule 1 method or by terminal anaesthesia for organ harvest.

Animals transplanted with cell and gene therapy products may be functionally assessed using motor function tests, cognitive tests and scans to confirm the restoration of their normal function. These tests are especially relevant to lysosomal storage disease models which exhibit musculo- skeletal, brain, cardiac and respiratory abnormalities.

Induced pluripotent cells may be assessed for their safety and efficacy by transplanting into immunodeficient mice. Occasionally certain induced pluripotent cells may induce tumours, and those animals will be sacrificed humanely before it exceeds the protocol severity.

Gene and cell therapy products which are specifically developed for treating tumours will be tested for their anti-tumour efficacy, after transplantation of the minimum number of tumour cells required to form a tumour. The time taken for the development of tumour may range between couple of weeks to a few months depending on the type of tumour studied.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Immunodeficient mice may develop pathogenic infections. Animals are therefore maintained in individually ventilated cages with sterilised bedding, water and food. Lysosomal storage disease mouse models may occasionally develop signs of Joint/muscular disease, respiratory, central nervous system or liver disease.



Animals that are conditioned using total body irradiation are expected to experience some weight loss and loss of condition during the first few days after irradiation. Pregnant dams that have had laparotomy for in-utero transplantation of embryos are expected to suffer moderate post-operative pain for a few days. Transplanted mice that undergo bone marrow biopsy are expected to experience moderate pain and transient difficulty in ambulation for a few hours. Superficial blood vessel sampling can (very rarely) cause profuse bleeding.

induced Pluripotent stem cells, upon transplantation can induce malignant transformation, and therefore some animals may form tumours, which can cause moderate pain. These tumours develop at the site of sub-cutaneous injection in flank region and is not expected to cause significant disruption to their normal activity. Animals that develop tumours are usually culled within a few days from the initial diagnosis.

Animals that are subjected to motor and behavioural function tests may experience some discomfort for a few minutes during each time point of testing.

Animals that are transplanted with tumour cells are expected to show weight loss, loss of appetite and general wellbeing for a few days, which may cause moderate pain. These symptoms usually relate to the tumour volume, site of tumour formation, degree of metastasis etc. On very rare occasions, superficial tumour may bleed or ulcerate and can cause secondary infections.

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

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

Most of the animals do not suffer from significant adverse effects from the procedures that are undertaken in this license. However around 10-20% of the animals may suffer some from some clinical signs for a short period of time, as a result of the underlying disease.

What will happen to the animals at the end of the study?

- Used in other projects
- Killed

Replacement

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

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

Primary immunodeficiency Diseases (PIDs) and Lysosomal Storage Diseases (LSDs) are very rare diseases, and patients are few (even at large centres like REDACTED). Affected individuals are often very young children from whom only small blood samples can be obtained. The use of animal models of these diseases also allows us to study the immune system in vivo which is often not ethical or practical in humans. No other non-animal models exist that mimic human immunodeficiency and lysosomal storage disorders diseases and allow xenotransplantation as well as the PID mouse models we want to use. The complex organisation of the haematopoietic system makes in vitro culture



unsatisfactory. In vivo experiments are required to measure engraftment and longevity of haematopoietic stem cells and development of functional immunity because the complex multifactorial interactions cannot be assessed in vitro. The safety of vectors over long periods of time and the potential for insertional mutagenesis can only be adequately assessed in vivo. In fact the current guide lines state in vivo testing is mandatory for testing efficacy of treatment in disease models, and assessing safety for the whole organism. It is not ethically possible to study PID, LSD, haematopoiesis or conduct cellular and gene therapy protocols in humans and current in vitro techniques are not sufficient for research or clinical regulatory bodies. In vivo experiments are required to measure engraftment and longevity of haematopoietic stem cells and development of functional immunity because the complex multifactorial interactions cannot be assessed in vitro. In addition we want to assess the impact on health and behaviour. The safety of vectors over long periods of time and the potential for malignancy can only be adequately assessed in vivo.

What was your strategy for searching for non-animal alternatives?

In vitro culture
Mathematical modelling

Why were they not suitable?

In vivo experiments are required to measure engraftment and longevity of haematopoietic stem cells and development of functional immunity because the complex multifactorial interactions cannot be assessed in vitro. In addition we want to assess the impact on health and behaviour. The safety of vectors over long periods of time and the potential for malignancy can only be adequately assessed in vivo.

In fact the current guide lines state in vivo testing is mandatory for testing efficacy of treatment in disease models, and assessing safety for the whole organism.

Reduction

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

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

For each experiment involving mice we will use statistical software packages and power analysis in order to define the minimum number of mice to be used as well as using our knowledge from previous experimental group numbers. Number of animals in each group will be adjusted depending on the effect size expected. Typically we expect experimental groups to consist of six to twelve mice. Sharing control groups for comparable treatments groups will also reduce the number of mice to be used.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Careful considerations were given to the Arrive guidelines and information available through the NC3Rs website using features such as Experimental Design Assistant to



ensure that the minimum number of animals are used in this project keeping in mind the experiments are robust and reproducible as possible and providing the best welfare possible for the animals.

What other measures apart from good experimental design will you use to minimise numbers?

Where possible, each animal will be used for multiple analyses post mortem (for example histological and immunological). Where animals have to be genotyped this will be done as early as possible and wild-type littermates will be used as controls thus minimising the need to purchase control animals. For longitudinal studies such as behavioural studies, we will maximise the observation points during the period when clinical signs are expected to reduce the overall 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We have chosen mice as we are studying models of inherited disease and mice are the most appropriate and best characterised lowest vertebrate group animal model. Murine models are regarded as the most appropriate judged by peer reviewed publications. Immunodeficient models are the most appropriate models which will allow the engraftment of human cells that are subjected to gene therapy and gene editing protocols.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Where appropriate, we use neonatal mice for studying haematopoiesis, by engrafting gene therapy and gene edited cells via intra-hepatic or supra-facial route in neonates and by inutero approaches by directly transplanting into foetuses.

However, some of the disease phenotypes are exhibited at certain developmental stages of the animal and often only in mature animals, requiring investigation at different time points.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Where possible we propose to treat before the onset of disease with in-utero injections rather than treat once disease has been established to minimise suffering. We have experience with breeding immunodeficient animals and with long established protocols that have well defined end points so are able to provide high level training and thus competence for experimental procedures. Where necessary we will seek out advice and expertise from other groups in the UK and abroad to minimise suffering.



What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The ARRIVE Guidelines

NC3Rs resources

Workman et al, British Journal of Cancer 102, 1555–1577 (2010) guidelines for tumour studies

How will you ensure you continue to use the most refined methods during the lifetime of this project?

NC3Rs website - <https://www.nc3rs.org.uk/>

Attending external conferences and seminars on animal welfare.

Explain the choice of species and the related life stages

We are interested in studying the disease mechanisms, and developing novel therapies for primary immunodeficiencies, metabolic diseases and malignancies in a paediatric setting. Therefore we have chosen to use immunodeficient models which are highly efficient in engrafting human haematopoietic and immune cells. Moreover, the mouse models we use faithfully reproduce most of the clinical signs and phenotypes observed in humans.

In general, we will use adult mice for most of the experiments. However, there may be a few instances where neonates and embryos may be studied, in order to specifically understand the pathogenesis around the onset of disease as seen in human neonates and the influence of a specific mutation in the perturbation of normal development of the haematopoietic and immune system.





99. Modulation of identified neural circuits for memory

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

The aim is to define the types of brain cells responsible for giving us the ability to create, store and recall memories. To investigate this, we will control the activity of select subpopulations of nerve cells while monitoring the activity from other nerve cells that are connected to them, focusing on brain regions that contribute to mnemonic functions, such as the hippocampal formation, and parts of the thalamus and basal forebrain.

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

Why is it important to undertake this work?

Creating, storing and recalling memories is fundamental for survival as it helps us anticipate novel experiences and make predictions on how to act. By studying these processes in rodents, which have very similar brain structures and nerve cell connections to humans, we can define how memories are distributed in the brain and gain insight into what happens when this process starts to break down in progressive neurological conditions such as dementia.

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



We are working on brain regions that are known to be affected in neurodegenerative diseases such as Alzheimer's disease, which include the hippocampus and entorhinal cortex. These are key areas of the cerebral cortex (the outer part of the brain) that are vital for memory, including how we find our way around and how we remember locations and events. Other brain regions below the cerebral cortex are also very important for memory. We will define the activity, kinds of nerve cells, and connectivity to these cortical regions to increase our understanding of how memories are stored.

We will compare data obtained from mice and rats (e.g. models of neurodegenerative diseases) to data obtained from dementia patients and healthy controls. These will include data on the distribution of proteins that mark different populations of nerve cells and the presence of pathological (dysfunctional) proteins that indicate the disease. Other data include new information on the activity patterns of the different kinds of nerve cells contributing to memory in connected brain regions.

Valuable scientific (neuroscientific and neurological) data will be collected, advancing our understanding of the mechanisms involved for creating, storing and recalling memories, discovering and defining new nerve cell types that are responsible for spatial/episodic memories that end up in brain regions such as the hippocampus, and also gaining much needed insight into how these processes break down in the stages leading to dementia.

Valuable data collected from animals will be published in open-access peer reviewed journals. The multidisciplinary approach we use to study the brains of both rodents and humans will benefit the neuroscience community as well as clinicians and patients through access to these data in published form but also via other outputs such as public talks and seminars, posters and social media.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The immediate benefits will be to the neuroscience and neurodegeneration fields. The outputs can be rapidly disseminated at conferences and in the form of preprints (e.g. BioRxiv) before peer review publication. Novel observations and new insights relating to the cell types critically involved in enabling mammals (including humans) to encode, store and recall memories will be used by other laboratories and scientists to advance their own research.

Our findings will benefit the public, e.g. through outreach events, explaining how and why we do research. If we discover how particular proteins implicated in neurodegenerative diseases directly affect nerve cell activity, this information will benefit patients, medical professionals and pharmaceutical companies. This may take a long time (3-5 years) due to the different avenues of investigation required to understand mechanisms underlying memories.

How will you maximise the outputs of your work?

Research results will be disseminated by publishing them in reputable journals in open access format, so that they are available to all

Results and knowledge will also be disseminated through public lectures and poster presentations in scientific meetings and in teaching



Open days, outreach events, science festivals

Collaborations with other groups will be used as a way to efficiently share knowledge and expertise

International exchange programmes, whereby scholars and students from other countries visit our research group

Social media and press releases, videos

Species and numbers of animals expected to be used

- Mice: 10,000
- Rats: 1,000

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Studying brain activity at the level of individual nerve cells, and in populations of nerve cells in different brain regions, requires surgery in order to implant miniature devices that can detect brain signals. These surgical procedures are similar to those done for human patients, in that rodents are given an anaesthetic to put them to sleep and block any pain, then the skull is exposed, and extremely fine wires are lowered into the brain and secured in place with special bone cement. Sometimes we will inject into specific regions of the brain a non-harmful virus that will express specific proteins in groups of nerve cells. Surgeries typically last 2 hours and the animal recovers in its home cage, often with its cagemates, within ~24 hours. Some more advanced surgeries will last longer (e.g. implanting more complicated recording/imaging devices) and have a slightly longer recovery.

For actual experiments, rodents have their memories tested by letting them do some behavioural tasks such as running in mazes for sugar rewards. For the animals that have undergone surgery, they will be connected to cables so that we can monitor brain activity on a computer while they do behavioural tasks, simply explore an environment, or sleep. In some experiments, we will shine light inside the brain using a fibre optic cable, and this light is able to activate the special proteins that are expressed by the virus that was injected during the surgery. This light activation causes the nerve cells to become activated and we can measure what happens to other nerve cells after light delivery. By implanting a mouse with a small plate attached to the skull, we can temporarily restrain them by the head while they are free to voluntarily move and rest on a treadmill. Mice adapt very well to this and it enables us to gain direct access to the brain using specialised probes (such as glass electrodes), in order to accurately record activity of nerve cells from small regions of brain that would be very difficult to target otherwise. In other experiments with mice, we can actually visualise brain activity through a microscope while the mouse is running and resting on the treadmill. This enables us to understand how brain activity of different populations of nerve cells relates to memory, such as spatial navigation and recalling events.



We will use genetically-altered animals that have been engineered to express human proteins that are implicated in neurodegenerative diseases such as Alzheimer's disease and frontotemporal dementia. The expression of these proteins in the brain affects memory, so that genetically-altered animals show deficits in memory that increases with age. We want to keep these animals for many months, including until they are 'aged' (greater than 15 months old), so that we can track how the brain circuits directly related to memory are gradually affected and understand the mechanisms involved in their degradation. For the vast majority of these genetically-altered animals, their daily life is unaffected and the memory impairments do not impact their welfare.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Although we give the animals appropriate anaesthetics for pain relief, after surgeries when the drugs wear off they may occasionally feel some pain where the implants have been attached on the skull. This is temporary and most animals return to normal within 1-2 days. However in rare cases where they exhibit clinical signs we would give appropriate drugs to reduce this.

To motivate the animals to do certain behavioural tasks like learning how to find a sugar reward in a maze (testing their memories), we may need to temporally restrict their diet. This means that they lose weight (typically up to 10% of starting body weight) while they are being trained and tested.

For aged animals (greater than 15 months) we expect some to develop age-related conditions, much like humans do. These conditions will be monitored closely and animals will be used for experiments at the earliest stage possible.

For a minority of genetically-altered animals that have been engineered to develop memory impairments, a particular line may show some minor impairments in motor coordination that increase with age (e.g. gradual loss of hindlimb movement). This is a consequence of the type of genetic alteration. REDACTED and wherever possible the scientific information will be obtained before significant motor impairments develop.

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

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

All animals that undergo surgery (~70% of animals) are expected to reach moderate severity, since the surgery involves exposing the skull and attaching implants. This experience typically lasts 2-5 hours over the lifetime of the animal (1-2 surgeries per animal). For the majority of its time under procedures, the severity will be mild or even sub-threshold (we find that the animals actually enjoy exploring mazes) and experiments where we record brain activity typically do not involve any anaesthesia.

What will happen to the animals at the end of the study?

- Used in other projects
- Killed



Replacement

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

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

The anatomy, neurochemistry and physiology of a particular region of the brain, or indeed individual neurons in a particular region of the brain, can only be established by the analysis of the brain itself and thus requires the use of animals.

It will be necessary to carry out the experiments on mammals rather than reptiles or fish because other groups of animals do not have a brain structure sufficiently similar to humans. Experiments will thus be carried out on rodents. With the increasing scientific interest and availability of genetically altered animals, particularly those that mimic some aspects of neurodegenerative disease, mice will be the preferred rodent species. Genetically altered mice will mainly be used to aid in the discovery and definition of particular populations of nerve cells, and to artificially change (increase or decrease) their activity, which can reveal how particular neurochemicals affect memory, and how this activity relates to disease mechanisms. We will endeavour to use the most appropriate model for neurodegenerative diseases such as Alzheimer's disease and frontotemporal dementia; these models may be replaced over the course of the licence so that we only use the 'best' currently available model(s).

What was your strategy for searching for non-animal alternatives?

In addition to studying nerve cells in human brain slices in a related non-animal project, care will be taken to monitor the progress of the use of in vitro methods, such as organotypic co-cultures, to determine whether they can substitute for at least some of the in vivo work. We are also keen to explore in silico methods (advanced computer models) by initiating collaborations with computer scientists.

It will be necessary to use invasive techniques to study the brain instead of non-invasive imaging techniques (e.g. fMRI, PET and other forms of imaging) because of the low spatial (millimetres or centimetres rather than nanometres) and temporal (seconds rather than milliseconds) resolution of these imaging techniques.

Why were they not suitable?

Although we will also study human brain tissue to compare to the rodent, this cannot replace the use of animals because the questions we ask require behaviourally-relevant activity patterns involving large populations of brain cells. These particular patterns can not be replicated in cultures either.

Reduction

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



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

We estimated the number of genetically altered animals generated through breeding and how many would be genetically altered (heterozygotes) and how many offspring would be non-transgenic. For animal experiments involving surgery (which includes the genetically altered animals), we based the numbers on previous licences involving similar procedures, the success rates for obtaining scientifically-relevant results, the difficulty of experiments, and the time investment in each animal relative to the number of researchers and their capacity.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Following expert statistician advice and taking assistance from the NC3Rs' Experimental Design Assistant, we can confirm that we have designed experiments compliant with subsection 4.1.3 of the Medical Research Council guidance with optimal sample sizes and high precision allowing within- subject and unbiased between-subject comparisons in order to achieve maximal statistical power.

What other measures apart from good experimental design will you use to minimise numbers?

We will monitor closely and implement efficient breeding, test new conditions using pilot studies and will be reusing tissue from the same animals for histological and electron microscopic investigations.

We will test antibodies as far as possible on tissue sections taken from the human brain.

Much of our work involves *ex vivo* analyses in which we perform immunocytochemical studies on perfuse-fixed tissue. In these experiments the sections that are not used are stored for future studies. Our multidisciplinary approach by combining physiology/tract-tracing/histology in individual animals enables far more data to be obtained from single animals than would be obtained employing standard methods. These combined experiments will be planned carefully for maximum scientific output with the minimum of cumulative effects.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The use of wild type and genetically-altered mice (and to a lesser extent, rats) will allow us to take advantage of the wealth of information already available, thus aiding the interpretation of our results. We will thus exploit the power of genetics (and, more specifically, of genetic alteration or transgenesis) to give us the specific or selective access to certain cell types that we need to carry out the proposed functional investigations.



Because of the wide availability of genetically altered mice and the relative ease of modifying mice, over the duration of the proposed licence more mice and fewer rats are likely to be used.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The cerebral cortex and related structures in the brain are relatively conserved in mammals. Rats and mice are the species about which we know most and are thus considered the least sentient vertebrate group in which to carry out the experiments. Data from these species are directly relevant to human cortical function and dysfunction.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Good surgical practice and aseptic surgery will be carried out using appropriate drugs to minimise any adverse effects of the surgery. Animals will be frequently monitored during post-operative recovery until being able to engage in species-specific behaviour.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

REDACTED

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We subscribe to the NC3Rs newsletter, attend Institutional/Departmental Animal Welfare meetings, attend animal welfare events (e.g. NC3Rs Research Days), and maintain excellent communication with animal technicians and veterinary surgeons.

Explain the choice of species and the related life stages

We are mostly using adult mice, and sometimes adult rats. They are excellent species to work with because we know a lot about their physiology and anatomy, which is very similar to human. They adapt very well to the experimenter (as with pets) and scientists have tools specifically designed to be able monitor brain activity that work very well with these animals. The vast majority of animal experiments in research institutions use mice (and rats), so the infrastructure (housing, husbandry, expertise of vets etc.) is optimised for their welfare. Furthermore, genetically-altered animals (mostly mice) have been bred that mimic some aspects of neurological diseases, such as Alzheimer's disease, so this makes these animals ideal as models for human diseases.





100. Molecular and Genetic Regulation of Glial Development and Function

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

Animal types	Life stages
Zebra fish	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 is the aim of this project?

Our work aims to understand what genes allow glia to develop properly and provide support to a healthy 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?

As life expectancy increases, there are a growing number of brain diseases affecting people.

Researchers need to continue figuring out how the brain develops and functions so that these diseases can be treated or cured. Our brains are made up of two types of cells — some of these produce electrical signals (neurons), and others provide support (glia). For centuries, research has focused on neurons being the cause of brain disease. In many conditions, including Parkinson's and Alzheimer's, the supportive glial cells are abnormal



and lose their ability to function. This research shows us that glia are very important in normal brain function. In fact, we now know that glia are often the underlying cause of these diseases. However, we still don't know much about how glia develop, and we don't understand how they protect our brains. Our studies try to start breaking the ice on how glia develop and function at a very basic level. We hope this will help find new ways to treat or prevent brain disease.

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

The goal of this project is to learn more about the fundamental principles giving rise to an appropriately shaped and functioning cell. This work will provide a valuable foundation for other research aimed at understanding the support cells (glia) of the brain. Some of the genetically altered animals generated in this research will also serve as valuable methods for designing new drugs used in the treatment of brain disorders. Throughout the project, we will share our findings with other researchers (at conferences or in collaborations) and will publish our results in open access journals.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

We currently collaborate with other laboratories in the UK and internationally, who will mutually benefit from our findings to improve their experimental designs and establish new research programs aimed at understanding neural diseases. In the future, our results will likely enhance the development of drugs and/or methods that prevent, diagnose or treat human disease.

How will you maximise the outputs of your work?

We already have active collaborations with basic and translational research labs that mutually rely on our research findings. We actively publish all of our results and openly share our methods and resources with anyone who requests them. As is required by most funding bodies and within the REDACTED, we publish our findings in open source platforms. Peer-review publication is often time-consuming and is often missing many negative results or unsuccessful approaches. We do and will continue to share our findings on open source un-reviewed platforms such as Researchgate and Bio Archives, which includes details of adverse outcomes and strategies in a public forum.

Species and numbers of animals expected to be used

- Zebra fish: 12950

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, adult wild type, mutant or transgenic zebrafish males and females are placed in a tank together and fertilised eggs are passively collected. We will also generate new



mutant and transgenic zebrafish lines by injecting genetic material into fertilised embryos and allowing them to grow to adulthood. These stocks will enable us to visualise the effects of mutations or collect physiological (i.e. normal living function) data in non-sentient embryos (i.e. embryos which are life stages that cannot feel pain). To preserve genetically modified zebrafish lines that are not being actively used, we will occasionally collect and store gametes (eggs and sperm) so that these lines can be re-established at a later date.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

In general, mutations with severe defects or malformations do not survive to sentient ages (i.e. in embryos up to 5 days after the eggs were fertilised). We do not expect that any of our mutation or transgenic modifications will adversely affect animals in any way. However, should any abnormal behaviour or clinical signs develop in growing or adult fish, they will be humanely killed.

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

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

Mild in all cases

What will happen to the animals at the end of the study?

- 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?

Unfortunately, we know so little about how glia develop, there are currently no alternatives to animals for our research. We are always working to replace some of our animal methods with other techniques. For example, we recently developed a CRISPR-based (a gene editing) method that allows us to screen genetic changes in early stage fish embryos before they can feel pain. This new method allows us to edit genes more accurately. It replaces older methods where large numbers of genetically modified adult animals were used and gives us the same results. We also actively collaborate with insect and cell culture labs. By sharing our ideas with these groups, we continuously evaluate other methods, including organoid culture methods. These efforts will allow us to continue replacing our animal methods as our research into glia progresses.

What was your strategy for searching for non-animal alternatives?

Cell culture (individual cells growing in a dish) and organoid development (a three-dimensional laboratory based culture method that enables the growth and analysis of whole tissues).



Why were they not suitable?

Cell and organoid cultures are being developed to study glia. However, these techniques have failed to replicate the mature cell shapes and functional qualities needed for our research. As we build our understanding of how glia mature and use this knowledge to adapt organoid culturing techniques, we will likely enable such studies in the future.

Reduction

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

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

The number of animals estimated for use in this project is predominately made up of breeding, maintaining, generating and storing genetically altered (GA) animals. We obtained these numbers by drawing on our experience carrying out similar studies. These estimates allow us to produce at least the amount of embryos we need for statically valid results.

Breeding and maintenance of GA animals: Breeding results in significant energy loss for the adult fish breeders. We, therefore, must ensure enough tanks of fish and prevent repeated use of breeders over a week. Some GA lines will be used more depending on the experimental design. However, we estimate that we will need up to 10 2.8L tanks per line (depending on the number of staff using each line). Overall, we expect to maintain up to 18 GA fish lines, expressing fluorescent markers or gene mutations, at any given time. Thus, the total number bred will be 18 GA lines x 10 tanks x 12 fish per tank. Stocks of breeders will be renewed every year, so the total fish number amounts to 18 lines X 10 tanks x 12 fish x 5 years= 10500 GA fish.

Generation of F0 founders: For our research, we plan to generate 18 transgenic and mutant lines. From experience, we know that the generation of genetically altered (GA) lines has relatively low efficiency and the presence of genetic modifications are not always passed to F1 offspring. To ensure that we successfully capture the desired mutation or transgenes, we will generate 5 F0 lines (with 2 tanks of each line) for each GA line we have proposed to make. Therefore, our usage is estimated as follows: 18 lines X 10 F0 tanks x 12 fish per tank = 2160.

Obtaining zebrafish gametes: For, obtaining gametes (sperm and eggs) it will be necessary to maintain separate stocks of males with optimal breeding performance temporarily. We will perform this for lines that need to be archived which from previous experience amounts to on average 6 GA lines per year. For a target of 2 tanks of 6 male-only fish (a multiplying factor of 2 has to be considered assuming a 1:1 ratio of males and females per clutch), we estimate 360 fish over 5 years.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?



All of our experiments are carefully thought out and follow the appropriate steps of the PREPARE guidelines checklist (https://norecopa.no/media/7864/prepare_checklist_english.pdf). Consequently, our experimental design relies on distinct hypotheses or questions. We use carefully selected negative and positive controls to ensure the validity of our findings. For example, phenotypes that we find in genetically altered embryos are confirmed by direct comparisons to control (un-altered) animals following the same test conditions. In genetic sequencing experiments, we extract both types of cells from the eye (the neurons- as a positive control, and glia – experimental cells). Most of our methods are already established and commonly used in zebrafish labs all over the world. However, when we develop new techniques, we carefully consider the literature and seek expert advice before starting. There are instances where we use blinding and randomisation to achieve unbiased results. For example, in reverse genetic screens, we use a random number generator to keep track of each gene we are testing. This way, the person imaging and quantifying phenotypes is unaware of which gene it relates to until the analysis is complete. All of our experiments are designed to use only the number of animals needed to generate enough embryos for each test and reach a robust statistical power. If we are uncertain about the analytical approach or power calculations, we always seek the advice of an experienced bio-statistician.

What other measures apart from good experimental design will you use to minimise numbers?

We also reduce the number of adult animals we use by collaborating and sharing some of our fish with other users at the REDACTED. We also maximise the outcomes of our findings by making our genomic data available on public databases. In turn, these open-source platforms provide other researchers with data and prevent replication of our work in other labs. We reduce the breeding of transgenic lines by using wild type embryos wherever feasible. We collect gametes from genetically altered adults and store them for later use to reduce the number of animals we maintain at any given time.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will be using non-genetically altered and genetically altered zebrafish lines. The latter were either previously made or will be generated by the proposed work. The purpose of this licence is strictly to create, house, maintain and store the zebrafish lines. There are no methods proposed that are likely to cause pain suffering distress of lasting harm to these animals.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?



All experiments that are outlined in this proposal will be conducted on non-sentient stages of development.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The maintenance and care of adult animals are carried out by highly trained bio-facility staff. Together with our animal facility staff and other zebrafish researchers at the REDACTED, we frequently discuss ways to improve animal welfare in the facility. This includes discussions about additional precautions for preventing diseases, enhancing the viability of embryos through diet and improving water quality by replacing old filtration equipment. REDACTED. We have shown that removing this very small amount of tissue causes no pain to our fish. This means our fish do not need to be held in a tank containing water to which an analgesic (pain killing drug) has been added and so enables us to return our fish to tanks where they can be fed and allowed to interact with others in normal water (see <https://www.liebertpub.com/doi/full/10.1089/zeb.2015.1165>). We will continue to work closely with these staff to refine our methods for our research.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We ensure our publications conform to the ARRIVE guidelines: <https://www.nc3rs.org.uk/arrive-guidelines>. We also make use of 3Rs tools that are specific to fish and continuously updated by the REDACTED biomedical services team: <https://www.ubs.admin.cam.ac.uk/3rs/search/category/species-specific/topic/fish>

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Our animal facility officers and staff continuously inform us of advances in the 3Rs as it relates to our work. All project holders are also required to have a member of their group attend quarterly facility meeting where these advances are discussed in more depth. Any implementation of these advances is done under the strict guidance of a named veterinary surgeon.

Explain the choice of species and the related life stages

Zebrafish are a classical developmental model that has been successfully used to understand many critical biological and disease-related questions in science. They are the best model organism for our research because they give us the ability to perform much of our experiments before they are sentient animals that can feel any pain. During these stages, zebrafish embryos are small and translucent which means that a microscope can be used to noninvasively observe normal and abnormal development.





101. Molecular architecture, function and dysfunction of the Blood Brain Barrier

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

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Our aim is to deepen the understanding of the mechanisms that link brain vascular malfunction and dementia with the hope that our results will be translated into the development of early diagnostic methods and 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?

A fine control of the brain environment is essential for the brain's function. Unlike other organs, the exchange of substances between the blood and the brain is tightly regulated by vascular and brain cells which together form the Blood Brain Barrier (BBB). The BBB provides cellular boundary that restricts the invasion of toxins and pathogens into the



brain, and controls the uptake of molecules from the blood that are necessary for brain function. Failure in BBB stability leads to severe neurological disorders. In spite of the crucial function of the BBB and the strong efforts made to understand the mechanisms that underlie its disruption, there are important aspects of BBB function that remain largely unexplored. How is the BBB maintained? How do the BBB cellular components interact? What are the molecules leading to BBB malfunction?

These studies will advance our understanding of neurological conditions like dementia. They will enable the development of new scientific tools and disease treatments by both identifying unknown BBB malfunction mechanisms and providing targets to design treatments to ameliorate or prevent dementia.

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

We will generate new information about how the brain vasculature is maintained and altered in disease. In particular, we will focus on understanding how different BBB cell types interact during health and disease. We will obtain information about what are the specific molecules involved in this process and how altering their amount can affect brain function and mouse behaviour. Our results will be published in scientific journals. The information we collect could be used in the future to design new drugs.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The most immediate benefits will be for the scientific community. This project will contribute to the scientific community, not only by generating new scientific knowledge, but also by preparing new high level scientists.

In the longer term, the results obtained with this research will benefit the public, by increasing the knowledge about the brain. It will also benefit neurological disease patients by contributing to the understanding of disease that eventually will be applied to the generation of new treatments.

How will you maximise the outputs of your work?

Throughout our research, we will establish collaborations that will allow us to get the most information with the highest quality.

Results will be shared in scientific meetings and also in outreach events. They will also be published in scientific journals and shared through other type of media available to the general public. Our publications will contain the positive and unsuccessful results so the scientific community can be informed and not perform unnecessary duplication.

Results will be also summarized in short videos that will be made available to the public.

The lab will have a website where we will publish the latest updates.

Species and numbers of animals expected to be used

- Mice: 7000

Predicted harms



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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The animals used in this project will undergo surgical interventions, injections of certain substances, behavioural tests and/or in vivo imaging. The experiments will typically last one month, from the initial surgery to the end of the experiment. During most of the experiment time, the animals will stay in their home cage. A lot of the time is waiting time for some genes or phenotypes to express.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

No severe effects are expected. They may lose some weight after the surgery, but pain will be prevented treating animals with analgesia. They could present abnormal behaviour like decreased activity. The effects are not expected to last more than 3 days.

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

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

The maximum severity for these experiments is moderate. It is estimated that about 80% of the animals may experience moderate effects like transitory sickness. The other 20% could be included under mild severity, since they won't undergo any major procedures.

What will happen to the animals at the end of the study?

- Killed

Replacement

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

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

The focus of the project is to study the interactions between the blood vessels and the brain cells in health and disease. Such interactions are dependent on the blood flow and the substances that are transported in these vessels thanks to the blood movement throughout the body. Currently there is no cellular model or computational tool that can resemble the complexity of such a system.

What was your strategy for searching for non-animal alternatives?

Cell lines of brain cells, induced pluripotent stem cells, organoids.



Why were they not suitable?

As stated above, none of the alternatives is able to resemble a completed circulatory system that irrigates brains cells.

Reduction

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

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

The number of mice has been calculated based on previous experiments that I and other colleagues performed at REDACTED, using the same techniques. For new experiments, power analysis will be used to calculate the appropriate numbers of animals in each group and professional statistical advice will be sought when required. Based on our experience of most of the models we can determine the group sizes required. As examples, to perform proteomics, each sample is generated from collection and pulling of protein of 8-10 mice. Each proteomics experiment needs to be repeated at least four times per condition. For studies in ageing animals the studies have to be overpowered to allow for a loss of animals in studies due to age-dependant adverse effects e.g. tumours. Most of the procedures are established and the variability of the models is known. However, with new approaches, pilot experiments may be necessary in small numbers of animals.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The experiments have been designed such that the animal work is reduced to the minimum. We will first collect mouse proteomics data. The results of these experiments will be used to test changes of the expression of the interesting protein candidates in cell culture models. This will dramatically reduce the number of animals used for candidate testing and will allow to select one or two candidates that will be further corroborated in animal models.

Whenever possible, already published data will be used to avoid unnecessary duplication of experiments. A good example of this practice is the use of already published proteomics and RNA-seq datasets.

What other measures apart from good experimental design will you use to minimise numbers?

We will use both females and males.

We will share tissues and experimental data that can be reanalyzed with collaborators.

We will revise our protocols, and reduce the number of animals if possible. For example, if by using a more sensitive mass spectrometer we can reduce the amount of animals needed for each proteomics experiment, we will do so.



We will try to get as much information as possible from the same individual. For example, in some instances, animals that have been used for in vivo imaging could be used for immunohistochemistry once the animal is killed.

Refinement

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

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Most of the experiments in this project will be performed in wild type mice. We will study molecular changes of the blood vessel - brain cell interactions in mice through ageing and in models in which neuroinflammation induced by administration of an exogenous compound. Occasionally we will include disease models in the studies, however, due to our interest in early disease alterations, we won't work with mice in severe stages of the diseases.

The surgeries will be performed under anesthesia and analgesia, and animals are expected to recover within few hours with no major complications. Nonetheless, mice will be monitored after every procedure to ensure there are no signs of distress like piloerection, abnormal locomotion or hunched posture. In addition, clear endpoints are defined for intervention either to correct weight loss and dehydration or for termination of the experiment if necessary.

Although the majority of our work will be conducted in vivo, there will be some phases of the programme in which multi-cellular culture models will be used to test specific molecular pathways that are identified with the animal work. We do not have the expertise to conduct these studies but we will collaborate with other groups that have proven expertise of combining in vivo and in vitro approaches to address specific research questions. In addition, the programme includes the use of ex vivo studies to probe cellular communication signalling in brain cells or slices. These approaches will reduce animal suffering given that the animal will be humanely killed before the in vitro experiment is performed.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The use of mice has many advantages over other models. Mouse is an ideal model to do translational research as there is a wealth of studies that compare mouse and human data. The understanding of astrocyte and endothelial cell biology is much deeper in mouse than in other species. This is an advantage towards reduction of the number of animals because already generated datasets can be reanalyzed. In addition, the AAV tools that we propose to use in this programme have been designed for mice and they do not exist for their use in any other species. Moreover, there is a wide variety of genetically modified models that we will use in future studies once we have defined the protein functions we want to explore.



What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

At the beginning, researchers will be exposed to the animals with frequency so they can learn the differences between normal and abnormal behaviour and appearance. Animals will be provided an enriched environment and they will be handled frequently to reduce their stress. When animals are required to perform a specific task, they will be familiarized with the new environment before the test. If surgical procedures are performed, we will do regular check outs, to assess recovery and pain. If there are signs of pain, analgesics will be provided.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Guidance on the Operations of ASPA, <https://www.nc3rs.org.uk/>

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Me and the rest of the people in the group will stay informed of the latest news by consulting resources as: ARRIVE and PREPARE guidelines, <https://www.nc3rs.org.uk/>, <https://www.gov.uk/research-and-testing-using-animals>, www.understandinganimalresearch.org.uk, www.frame.org.uk and <http://www.procedureswithcare.org.uk>.

Vets will also be contacted with frequency to keep improving our practice.

Explain the choice of species and the related life stages

We decided to use mice for several reasons:

They have a complex mammalian brain which allow us to study the mechanisms we are interested in, and further translate our discoveries into human.

There is a wealth of published data from many different mouse models that we could use to understand our observations.

There is an enormous number of tools for mouse work and genetic models that we can use for our studies.

Most of the experiments will be performed in young adult mice. A subset of experiments will be performed in young and old mice, to better understand the effects that age has on the brain vasculature.





102. Molecular Basis of Development and Disease

Project duration

5 years 0 months

Project purpose

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

Key words

No answer provided

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

Retrospective assessment

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

Objectives and benefits

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

What is the aim of this project?

Our aim is to understand how genes regulate developmental processes and the maintenance of stem cells and organ formation during embryonic development as well as in adult organisms. We also aim to understand how deregulation of these processes leads pathological conditions

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The findings from our research will provide information on how organ formation takes place during embryonic development and how stem cells are maintained in organs such as brain and intestine. This information will have applications in developing better methods of cell reprogramming, tissue engineering and repair as well as increase our understanding of cancers. In addition, deregulated developmental processes can give rise to pathological states such as neurodevelopmental disorders, neurodegenerative diseases and cancers.



Mouse models for developmental and disease processes will help unravel disease mechanisms and have applications in evaluating therapeutic approaches.

What outputs do you think you will see at the end of this project?

Research conducted under this PPL will

provide new information on the mechanistic understanding of neuro-developmental and late onset neurodegenerative diseases which will be communicated for publication in high impact peer reviewed journals.

be presented at International and national conferences and workshops

Expected benefits from : studies on how knockout of/or mutations/deletions in proteins that form the basal body of the primary cilia cause neuro-developmental disorders (ciliopathies) and motor neuron disease.

What we hope to find from our research:

The molecular changes associated with mutations in basal body proteins such as Talpid3 seen in the ciliopathy-Joubert syndrome (a neurodevelopmental disorder).

How mutations in basal body proteins such as Nek1 and C21orf2 lead to motor neuron disease (a neurodegenerative disorder)

The molecular mechanisms that links stem cells and neurodevelopmental/neurodegenerative disorders and find out if these diseases share some common mechanisms.

Expected benefits from :studies on how DNA binding proteins such as Caudal, Polycomb group and Trithorax group regulate stem cells in the brain and intestine.

What we hope to find from our research:

Studies such as the ones proposed for Cdx, PcG and Trx-G genes will provide new insights into the molecular basis of the regulation of stem cell identity and function in adult tissues such as the intestine and brain. The outcomes will have implications in our understanding of tissue regeneration with implication for tissue repair and tissue engineering.

Data from such *in vivo* experiments will enable us to design and perform *in vitro* experiments to dissect the molecular mechanisms of the function of these genes.

Experiments such as those on the skeletal patterning mutants will provide an understanding of the molecular basis of human birth defects which are developmental lesions.

Expected benefits from: What is the molecular basis underlying the pleiotropic role of Angiogenin in neural development, neurodegeneration and stem cells.

Studies proposed under this objective will provide new insights into the molecular basis of the regulation of stem cell identity and function in adult tissues such as the intestine and



brain. The outcomes will have implications in our understanding of tissue regeneration with implication for tissue repair and tissue engineering.

Data from such *in vivo* experiments will enable us to design and perform *in vitro* experiments to dissect the molecular mechanisms of the function of this gene and the mutations found in motor neurone disease patients.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The proposed research will lead to a better understanding of (1) stem cell regulation; (2) ciliopathies, a group of neurodevelopmental disorders classified as rare diseases which are ill understood and; (3) late onset neurodegenerative diseases.

Our research findings and mouse models will

be of interest to the rare diseases patient interest group be of interest to patients with motor neuron disease have applications in therapeutic evaluation.

have implications for regenerative medicine and directed differentiation with applications in tissue repair, regeneration and wound healing.

will help develop better approaches to their treatment of neuro-developmental and neurodegenerative diseases

The genetically modified animals will provide disease model systems which will be of interest to pharmaceutical companies for evaluation of therapeutics.

Our research will also be of interest to clinicians and pharma.

How will you maximise the outputs of your work?

Based on our findings we will seek to collaborate with tissue engineers and clinicians for appropriate applications of our findings on stem cells, motor neuron disease and ciliopathies.

We will present our findings in scientific conferences and also highlight any unsuccessful approaches.

Successful application of the 3Rs will be disseminated through NC3Rs. We will publish our finding in peer reviewed journals.

Species and numbers of animals expected to be used

Mice: Estimated number for mice annually on an average is 3110 mice which will include the wild type mice generated through breeding protocols

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Most of the strains we propose to use have already been generated by us or others. We will be generating a few lines with human disease specific changes. For this we will inject hormones, obtain very early embryos or eggs and inject these eggs/embryos with genetic material, transfer them to foster mice and allow the embryos to come to term. These mice will be checked to see if they carry the altered gene and bred for experimental purposes as described below.

Pre-implantation stage embryos obtained by superovulation or sperm will be used for cryopreservation.

Embryonic stem cells will be derived from pre-implantation embryos isolated after superovulation.

More typically we will mate genetically altered mice and obtain embryos or tissues after humane killing of the mice. We may for some strains induce gene expression by either administering Tamoxifen or tetracycline and then isolate tissues or embryos after Schedule 1 killing.

The cells, tissues or embryos will be analysed for cellular, molecular and biochemical changes using histology, immunohistochemistry, fluorescence activated cell sorting for cell cycle analysis, proteomics and transcriptomics.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Some of the DNA binding protein knockout mice are smaller than their normal litter mates, exhibit vertebral identity transformations, show stem cell defects in the brain and gut. They groom and feed normally and we typically kill these by schedule 1 method at the first signs of distress, weight loss or pain.

Some of the DNA binding protein knockout mice are similar in size to their normal litter mates. They groom and feed normally. They have vertebral defects and stem cell defects in the gut. We typically kill these mice by Schedule 1 method at the first signs of distress, weight loss or pain.

The genetically altered basal body protein mutant mice show signs of ataxia at around post natal day 15, such as righting themselves slowly in comparison to their wild type litter mates and starting to drag their hind limbs. They also start to show a domed head at around day 15. However, they are able to groom themselves and are able to suckle. These mice are killed before the ataxia progresses ie at postnatal day 15 or earlier. Beyond 15 days the ataxia progresses and animals are in distress by day 20.

For the new strains that we propose to develop for patient specific motor neuron disease/ciliopathy risk factors and for their knockouts we cannot predict the severity of the phenotype until they are generated. Once generated these animals will be monitored regularly for adverse effects and killed by Schedule 1 method if they show signs of distress.



One of the knockout mouse strain we propose to obtain from a collaborator shows no overt pathological phenotypic changes. These mice breed and feed normally but mice over 9 months of age develop leukopenia. In this case the mice will be killed by Schedule 1 before the onset of this phenotype.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Under breeding and maintenance of GAA, all the Reporter transgenic strains such those expressing epitope tags, fluorescence tags, the Cre driver strains and conditional knockout mice with LoxP sites that we will use have no adverse phenotypes and so fall under the mild severity band. This is because they are not expressing any harmful mutations.

Currently 25% of mice from five of current genetically modified REDACTED show moderate severity as either they have gut or brain defects.

What will happen to the animals at the end of the study?

- 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?

Organs are not homogenous populations of identical cells but are heterogeneous and have a 3-D organization which depends on complex cell interactions. Hence, any analysis of the co-ordinated regulation of proliferation, differentiation and tissue patterning in any organ needs to be carried out in developing embryos and adult organisms. This is because such processes occur within a defined anatomical three dimensional tissue architecture containing the multiple cell types. Similarly, in order to study stem cells *in situ* and tissue homeostasis, an animal model is most appropriate.

What was your strategy for searching for non-animal alternatives?

Wherever possible, for evaluation of gene constructs and preliminary studies, we already make use of tissue culture models. We use embryonic stem cells to evaluate our CRISPR/Cas9 strategies prior to applying them to mouse embryos.

We have also been developing 3-D organoid models for carrying out biochemical studies using human induced pluripotent stem (iPS) cells or mutant mouse ES cells. I have been funded by a project grant for developing patient specific induced pluripotent stem cells as a disease model to reduce and replace animal models of motor neuron disease. We are also developing brain organoids from human iPS cells as a model for Frontotemporal dementia.

We also use early zebrafish embryos (stages upto 120hpf which are not regulated) for gene construct evaluation.



Why were they not suitable?

Tissue cultures although excellent to carry out biochemical and molecular biological characterisations of the functions of genes and genetic pathways are not entirely appropriate for our studies.

In undertaking studies such as on mammalian development or to study late onset neurodegenerative diseases which cannot be fully recapitulated in cell cultures we have had to consider the use of laboratory mice.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The KO mice will be maintained as heterozygous lines. To generate homozygous experimental animals heterozygous crosses will be set up which will produce offspring in Mendelian ratios ie 25% will be homozygotes, 50% will be heterozygotes and 25% wild type.

The Transgenic fluorescent reporter, cell cycle reporter and Cre deleter mouse strains will be maintained as hemizygous lines.

The conditional knockout mice ie floxed mice will be maintained as homozygous lines.

How we have arrived at the proposed number of 6 mice of each genotype for phenotypic analysis.

For the new GAAs that we will generate and for the crosses with reporter or Cre strains of mice, we cannot fully estimate the phenotypes and standard deviations of the intercrosses. Hence we have used a crude method of calculation based on the law of diminishing return.

This method is called “resource equation” method. This method is used when it is not possible to assume about effect size, to get an idea about standard deviation as no previous findings are available or when multiple endpoints are measured or complex statistical procedure is used for analysis.

According to this method a value “E” is measured, which is nothing but the degree of freedom of analysis of variance (ANOVA). The value of E should lie between 10 and 20. If E is less than 10 then adding more animals will increase the chance of getting more significant result, but if it is more than 20 then adding more animals will not increase the chance of getting significant results. Though, this method is based on ANOVA, it is applicable to all animal experiments. Any sample size, which keeps E between 10 and 20 should be considered as an adequate. E can be measured by following formula:

$E = \text{Total number of animals} - \text{Total number of groups}$



In our case $E=12$ (6+6) and total number of groups is 2 with $E=10$ and therefore the numbers we have proposed is adequate. Once we have the mice and analysed the phenotype we will be able to do power calculations to confirm that these numbers are sufficient.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Since we are using GAA for our experiments it is not easy in all cases to predict the numbers we will generate simply because we will not only generate homozygotes and wild types to be used in our analysis but also heterozygotes in the case of our mouse strains show no phenotype.

For each strain, the breeding nucleus is small consisting of 2 breeding pair replaced every 4 months.

For the functional and histological assays we propose to compare at least 6 homozygotes from postnatal, adult and aged mice with their wild type (WT) type littermates if the mutation is fully penetrant as is the case with most of our strains.

What other measures apart from good experimental design will you use to minimise numbers?

To minimise the use of mice and mouse embryos for preliminary studies we use cell cultures and embryos of lower vertebrates such as zebrafish (stages up to 5d post fertilization which are non- regulated). The findings will then be confirmed using the mouse so that we only have to use the most appropriate and minimal number of constructs on a small number of mouse embryos and sacrifice animals at only the required stages.

Our breeding nucleus is generally small between 2-3 breeding pairs and colony numbers are managed efficiently to keep mouse numbers low.

We will also use refined technologies such as the Crispr/Cas9 system to generate knockdowns/knockouts and transgenic mice as these are emerging as more efficient technologies which will reduce the use of animals.

At appropriate places in the experimental approaches, I have indicated the *in vitro* techniques that will be used in conjunction with the animal experiments to minimise use of animals.

All of the mouse experimental approaches used are standard and well established techniques in my laboratory so failure of experiments is not very common.

We have a dedicated Statistician who we will consult, although the current developmental biology experiments we propose do not involve complex statistical evaluations.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.



Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Models: The mouse has been chosen as the model in this study as it is the lowest vertebrate group in which studies of organogenesis, stem cells and patterning can be carried out as they share many similarities with humans. The developing mouse embryo is an extensively used model system for determining the role of normal and mutant genes in developmental processes.

The mouse embryo is a good model system for understanding vertebrate development for the following reasons:

well characterised genetic markers are available and the information from genome sequencing allows identification of functionally active genes

the gestation time of the mouse is reasonably short

conditions have been well worked out for the isolation and culture of pre-implantation and early post-implantation mouse embryos

it is possible to introduce new or modified genetic information into the pre-implantation and early post-implantation embryos that could perturb development or serve as markers to trace the cell lineages and follow expression of a particular gene product

it is possible to introduce the normal or genetically modified embryonic stem cell (ES) or cells from the inner cell mass into mouse blastocysts in order to generate injection chimeras and eventually derive transgenic mice that carry an altered gene.

The mouse models we are using to study the role of the basal body/ cilia proteins are conditional knockouts which are either temporal or spatial knockouts thereby minimising the number organs or stages that are affected.

Since we are investigating cellular and molecular mechanisms we use animals at the preclinical and the stages showing the earliest signs of clinical manifestation thereby minimising suffering and distress.

Methods

The majority of our methods for analysing the mutant mice are of mild severity which either involve isolating fetal forms or tissues from post-natal, juvenile or adult mice by Schedule 1 killing to analyse the cellular, molecular and biochemical changes. These cause minimal suffering and distress.

Our methods for investigating cell cycle/proliferation and protein synthesis is similarly are minimally invasive.

Conditional gene expression is also a refined approach to understand gene function since we turn off/on the gene only in specific cell types or at specific times in development or life cycle.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?



We already use zebrafish embryos (up to d5 post-fertilization) or cell cultures to evaluate constructs and cellular phenotype prior to generating transgenic mice. Organs such as the cerebellum develop post-natally and intestinal epithelium matures post- natally and in such cases post-natal mice are the best models .

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Mice will undergo minor surgery for vasectomisation or during embryo implantation under anaesthesia and are expected to recover quickly and will be given painkillers and post operative care just like people recovering in a hospital. They will be monitored regularly to ensure that they are not suffering from any infection or problems with wound closures.

We will use cell type and tissue specific knockouts i.e. conditional KOs to minimise the phenotypic consequences of gene deletions or over-expression.

Stock lines which show no adverse phenotype as for example - Reporter or Cre expressing mouse strains are maintained as homozygous line. Other strains which show adverse phenotype in the homozygous state are maintained as heterozygous lines. These contribute to maintaining smaller number of mice in the colony.

New mouse mutant lines that we will be generating will have very well defined monitoring scheme to identify stages and extent of suffering and distress.

In the case of newer transgenics or KOs REDACTED yet to be generated, we will consult with the NAWCO, NVS and Home Office inspector to decide on the steps for minimising distress and a humane end point that will allow functional analysis without undue distress to the animal.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will use the Experimental design assistant and other resources from the NC3Rs to ensure that our experiments are conducted in the most refined and effective way. We will also use the updated ARRIVE guidelines in our reporting

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We use the information in the NC3Rs website to inform us of best practices in terms of the 3Rs.

Explain the choice of species and the related life stages

Our aim is to understand molecular basis of stem cells homeostasis, neuro-developmental and neuro- degenerative diseases and the mouse is a good model system as it is a mammal and techniques to manipulate its germline are well worked out. In addition, aspects of neural development are similar.

For studying genes that regulate development, stem cell identity and maintenance, in self renewing tissues as intestine and on organs such as the brain, we will use embryonic , peri



and postnatal stages as well as adult mice from strains which have been genetically modified. Such studies will provide an understanding of how these genes regulate developmental processes.

For studying genes that have been shown to be mutated in motor neuron disease by Genome wide analysis studies (GWAS) we will use post natal, juvenile and adult mice which have been genetically modified. This will enable to study early molecular and cellular changes before the overt manifestation of the disease.





103. Molecular Regulation of Mammalian Development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Mice	juvenile, adult, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The overall objective of this project is to increase our understanding of the molecular processes regulating mammalian embryo development. Particularly, we want to study the function of specific genes involved in this process.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The discovery that certain factors can 'reprogramme (transform)' mature cell types into stem cells holds great potential for new biomedical applications, such as cell replacement, drug testing and disease research. Reprogramming allows us to turn any cell of the body into a stem cell. However, the mechanisms involved in this technique are only just being identified and the success rate of the method remains very low. One way to improve our understanding of reprogramming is to study the natural programming mechanisms that begin after fertilisation in the mammalian embryo. In addition, basic scientific discoveries resulting from this programme of work will provide knowledge that would benefit couples undergoing reproductive technologies such as in vitro fertilisation.

What outputs do you think you will see at the end of this project?



We aim to study the onset of the gene expression event in mouse embryos. We aim to uncover new factors that control early embryogenesis and further investigate their roles during the embryo has the potential to become all the cell types in the body (totipotency). This work will bring novel information about gene function at the beginning of development

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Identification of molecular mechanisms will lead to a greater understanding of how totipotency is achieved/maintained and has important implications for reproductive technologies and areas such as regenerative medicine.

The long-term potential benefits of this study are that data generated may have far-reaching implications for the treatment of infertility conditions and in the field of regenerative medicine.

How will you maximise the outputs of your work?

Findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings. We are preparing a major publication based on the results from the previous project and work has been presented at national and international conferences. The transgenic animals developed will be valuable and made available to other scientists.

Species and numbers of animals expected to be used

- Mice: 13,100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Our project includes hormone Intraperitoneal injections. For in matured oocyte collection, only one hormone (PMSG) injection needed. To induce super ovulation, a second hormone injection (hCG) will apply 48 hours post PMSG inaction.

We will perform surgical procedure in the “Embryo Recipient” procedure.

Duration: 15-30 mins per recipients.

Procedures:

Recipients for embryo transfer will be rendered pseudo-pregnant by mating with a vasectomised sterile male

Under general anaesthesia a single/bilateral flank or midline laparotomy will be performed and genetically modified embryos will be implanted into the oviduct or uterus (AB-G).



Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

In order to provide eggs and early embryos, female mice will be given hormones to maximise the number of eggs and embryos produced. They are then killed by a humane method and the embryos collected, usually one or three days later. During the procedure these animals will only experience transient pain at the time of injection. Using molecular laboratory techniques we will identify and characterise the key factors involved in the regulation of gene expression during early embryo development in vitro – so the vast majority of the mice we will use are bred and killed for egg and early (up to day 4) embryo harvest and not subject to any invasive procedures.

The ability to alter specific genes in the laboratory provides researchers with the opportunity to study the function of a particular gene. We will culture the eggs and embryos in specialist culture systems; assess their development and the effect of modified gene function. Very occasionally we may need to perform surgery on the mice, under general anaesthesia (for example to transplant some embryos into female mice or to perform a vasectomy on male mice). These are essential techniques (with moderate severity) but good surgical techniques, anaesthetic and pain relief will be used during and after surgery to minimise adverse effects. The mice are allowed to recover and will be monitored closely post-operatively. Some mice will deliver at term and other pregnant females will be humanely killed at specific time points in early gestation. At the end of the study all the mice will be humanely killed by an approved method.

We will also breed genetically altered (reporter) mice (to obtain eggs and early embryos). These mice have been generated under other project licences. The effects of the genetic alterations (a fluorescent marker tagged to a protein of interest) are negligible, and the animals suffer no adverse side effects of this alteration. These mice will be superovulated and mated to obtain early embryos, experiencing no more than the same minor discomfort at the time of injection, as the control mice described above.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

The breeding and maintenance of genetically modified animals (Protocol 4) should not generate any severity level.

All mice undergoing superovulation (Protocol 1) will experience mild severity levels.

All mice used as embryo recipients will (Protocol 3) will experience moderate severity levels.

Protocol 2 involves the manipulation of oocytes/embryos up to the blastocyst stage

What will happen to the animals at the end of the study?

- Killed

Replacement



State what non-animal alternatives are available in this field, which alternatives you have 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 will use mice mainly as oocyte/embryo donors with the remainder of the experimental work being carried out in the laboratory in vitro. There is no alternative to the use of animals for the basis of our work but in silico analysis will be used in order to minimise the numbers of animals used. We cannot obtain oocytes or embryos without the use of live animals.

What was your strategy for searching for non-animal alternatives?

Il proposed studies build on extensive data derived primarily from in vitro and biochemical studies carried out either by this group or by the international scientific community.

Why were they not suitable?

See above.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Wherever possible, attempts will be made to reduce the number of animals used in order to address specific scientific questions, while making maximal progress in achieving the goals of the project. Experimental design is given priority, with regular consultation between all members of our collaborative groups. Numbers of animals used in each experiment are based on estimates of variability expected from our previous experience or from published data. Appropriate positive and negative control treatments are included where necessary. All procedures are carried out by highly- trained staff to maximise the success of vulnerable procedures such as oocyte/embryo microinjection and nuclear transfer.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Oocytes and zygotes will be obtained from superovulated mouse donors. Short interfering RNA (siRNA) molecules, DNA templates, cells or sperm will be injected into the oocytes/zygotes by microinjection. Negative control groups will consist of either a scrambled siRNA for the target gene of interest or a non- injected group/sham injected group, depending on the experiment. Previous experience using this technique in REDACTED has shown that at least 30 embryos should be successfully injected for each group. This number is obtained from a power analysis calculating that a sample size of 30 embryos per group will permit observation of a 5% significant difference in measured values, based on prior standard deviation. The concentration of each siRNA will be titrated to ensure that the lowest concentration is used to deplete gene function in order to prevent off target effects. As these animals are superovulated the experiment will be repeated



three times on different occasions to avoid bias from any one single animal. Differences in gene expression will be assessed by quantitative PCR (data analysed by Student's t-test and ANOVA). Protein levels will be measured by Western Blotting and/or immunofluorescence, or biochemical methods – such as chromatin/methylation analyses. Nuclear transfer experiments using pre-treated donor cells to alter the chromatin structure of the DNA of the donor cell before transfer may increase the efficiency of current nuclear transfer protocols. We propose to use immunocytochemical assessment of the chromatin in potential donor cells and molecular analyses of selected marker and reporter genes to study reprogramming efficiency of candidate chromatin remodelling proteins. The in vitro manipulation of chromatin status of different donor cell types may identify favourable epigenetic markers that would allow these cells to be sorted and used as nuclear donors, eliminating some of the 'lottery-effect' of choosing cells for embryo reconstruction. In most cases, the number of oocyte and embryo donor animals used will be minimised by superovulatory treatments, which increase the numbers recovered by approximately fivefold. We will also use molecular techniques that allow assay of single oocytes and embryos (and in some instances single blastomeres) reducing the degree of replication and numbers of observations required.

What other measures apart from good experimental design will you use to minimise numbers?

Careful statistical treatment of all data will be undertaken, gaining as much information as possible from each experiment. Whenever feasible, experiments will be consecutive to allow dissemination of results from previous experiments to influence current experimental design. A priori power analyses will be conducted prior to new experiments to determine an appropriate sample size to achieve adequate statistical significance.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The mouse is selected as a model species for these studies for several reasons. Firstly, there is more information available about this species than any other in genetics, molecular biology and reproduction. Secondly, the short reproduction interval allows studies to be completed more quickly than in any other Page 7 of 7 mammal. Finally, more consistent observations can be expected as inbred strains are maintained in a closely controlled environment. All mice undergoing surgery will receive pain relief (analgesia) and good post-operative care. Moreover, all of our animals are housed under pathogen free, environmentally controlled conditions. Animals are routinely monitored for the presence of pathogens that could potentially lead to infections.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The age we will use is the standard.



What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

No answer provided

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We have adapted the most refined method to induce ovulation (Takeo and Nakagata, 2015 PLOS One).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will check related references, attend 3Rs meeting and discuss with other researchers.

Explain the choice of species and the related life stages

Mice : The mouse is selected as a model species as there is more information available about this species than any other in genetics, molecular biology and reproduction.

To investigate fertility issues we need adult mice to produce the oocytes/embryos we need for our experiments.





104. Morphogenesis of the early mammalian embryo

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

The aim of this project is to understand how mechanical forces shape complex three-dimensional structures such as tissues and organs out of simple cell populations using the early mouse embryo.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Birth defects are one of the leading causes of infant mortality across the world, many of which can be identified early in pregnancy, however we have only limited ability to repair such defects in the womb. For adults, hundreds of thousands of people who are in need of new organs wait on long transplant lists, relying entirely on donors, and face the possibility of having to take immune-suppressant drugs for the rest of their lives. The options available to these patients would greatly expand if we were to one day be able to grow or generate new tissues and organs outside of the body or in a dish. While we have made significant advances in understanding how genes are regulated throughout development, and using stem cells and organoids can differentiate specific cell types and populations,



we are not yet able to build more elaborate structures such as organs or even multi-layered tissues.

The work we propose will allow us to determine how the early embryo grows and develops from simple, homogeneous starting materials; how cells are organized to form tissues, how tissues coordinate to create layers and shapes, and what mechanical forces are required to fold these tissues into a functioning, moving organ such as a beating heart. Only with this knowledge can we begin to replicate these processes ourselves, using cell culture or organoids to generate tissues and complex shapes, to know when and where to manipulate and what to add and what to subtract. The ability to one day repair damaged organs, treat developmental defects in utero, and even grow whole, functional organs outside of the body will depend on our understanding of how the embryo builds such complex structures.

What outputs do you think you will see at the end of this project?

The successful conclusion of this project will result in at least several publications, collaborations, and the generation of a number of datasets and models that will be shared freely with the scientific community. Information gained from experiments will be used as a platform for further, more advanced studies that will continue to build off of the previous goals. Our insights and analysis from studying foregut formation will reveal mechanisms that are likely re-used throughout the development of a wide range of tissues and organ systems.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short-term, datasets that are generated may be shared with collaborators or public databases to be used freely by the scientific community. At the completion of this project, all datasets, computational tools, technical drawings and specifications, computational models and tools used to generate publications or demonstrate advances will be shared openly with the scientific community. The knowledge gained from this project will enable researchers in other fields such as gastruloids or organoids to employ new techniques to make more complex structures and further advance the development of their systems. Additionally, advances in imaging and embryo culture resulting from these studies will allow researchers to study later and more complex stages of mammalian development.

How will you maximise the outputs of your work?

We will actively seek collaboration with other researchers who may have complimentary knowledge or may directly benefit from our studies, as well as ensuring all results and data are made freely and publicly available. Results will be shared both in publications and at conferences and meetings that are widely attended by the scientific community. Public engagement activities will help dissemination of the knowledge to the general public.

Species and numbers of animals expected to be used

- Rats: 300
- Mice: 5000

Predicted harms



Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Adult mice will be used to produce embryos either through natural breeding, super-ovulation which involves the injection of hormones at two timed intervals, or through embryo-transfer experiments where female mice are anesthetized and one or two small incisions are made in their back order to place embryos inside the uterus or oviduct. The majority of mice used will experience only no amount or only very mild pain or discomfort. Embryo transfer and super-ovulation protocols are only occasionally required, and the majority of experiments will rely on natural breeding of genetically altered mice to produce embryos, and before recovering those embryos the female is first humanely euthanized and experiences no pain or discomfort.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Only mild, temporary pain or discomfort is expected for all animals on this project, with the highest degree of pain experienced for animals undergoing vasectomy or embryo transfer procedures. These effects are temporary, lasting no more than a day or two and can be managed with pain medication and animals will be monitored like a human patient under a doctor's supervision, and are not expected to have any long term physiological or behavioural effects. Complications from these surgical procedures are very rare in our experienced animal facility.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Only vasectomy and embryo transfer procedures are expected to produce an uncomfortable degree of pain and discomfort, which is only temporary and will be managed with pain medication. Only a small number mice will be used for these procedures, and most will only experience either no or only a very mild amount of pain or discomfort. All other mice used in this project involve the breeding of males and females, which is not expected to cause any undue level of stress.

What will happen to the animals at the end of the study?

- 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 understanding of how an organism develops from a single-cell of limitless potential, to all the complex cells, tissues, and organs that comprise an adult animal relies on our ability to observe and interrogate this process in nature. Mouse embryos are a close analog to human development, sharing many of the same genes and processes that shape organs and tissues. Birth defects seen in humans are also found in mice, allowing us to study these processes in the hopes of one day being able to correct them in the womb.

What was your strategy for searching for non-animal alternatives?

Because we understand so little about mammalian development, there are no synthetic or artificial alternatives that can replace actual embryonic development. Research into gastruloids and organoids are the closest non-animal analog, however this field is still in a very young stage, and indeed would stand to benefit from the results of this project.

Why were they not suitable?

The generation of gastruloids or organoids can make approximations of some cell types or structures, but they do not form embryos or even functional organs, nor can they be used to validate "normal" development as we do not yet even understand what "normal" development is.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 was estimated from similar experiments performed over the past five years, taking into account the rate of failure, the frequency of false pregnancies, and the estimated number of embryos and observations that are needed in order to make statistically sound conclusions. The number of experiments needed to ensure confidence in observations and measurements is also guided by accepted numbers commonly used in literature. More conventional power calculations are not suitable in this case as much of the work is based on discovery and observation or measurements in an inherently stochastic system. It is not appropriate to employ standard deviation as a measure of confidence in a system where that standard deviation may instead represent the normal, natural variation.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

While it is impossible to control whether or not animals become pregnant nor the number of embryos they produce, we are able to estimate based on expected rates of failure generated from years of previous data for similar experiments, combined with the NC3R's Experimental Design Assistant where appropriate, the number of animals that would be required to confirm observations and measurements in a statistically significant way.



What other measures apart from good experimental design will you use to minimise numbers?

Ensuring a controlled breeding environment will help to reduce the number of false pregnancies. In certain cases, ultrasound can be used to verify pregnancy, reducing the number of females that are sacrificed unnecessarily for each experiment. Additionally, females used for breeding and super-ovulations can be re-used in the same or other protocols. Continuous data analysis on results as they are obtained will allow us to continually refine the number of animals that are needed in order to achieve a statistically significant result.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We use mice as our model organism to study early mammalian development. The vast majority of our protocols are designed to only cause minor, temporary, or no pain or discomfort to the animal, and no more than is expected to be caused by natural breeding behaviours. In the case of more severe procedures such as surgeries to implant embryos, or vasectomize males, these are one-time experiences, which will be closely monitored by trained animal staff and technicians in order to minimize any pain or discomfort experienced by the animal.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice are a close, non-primate analog to human development, and an excellent model organism that has been well-studied for decades. Our understanding of mammalian development is so limited that there is no viable alternative that would achieve the same amount or quality of results.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals will be closely monitored whether it is for surgical procedures or during the course of normal breeding and husbandry. Changes in breeding behaviour for example can indicate a change in environmental conditions that causes stress for the animals, which can be identified and corrected. Local best practice guidance will be used unless stated. This guidance is reviewed annually by the AWERB and experienced NACWOs and veterinarians and reflects best practice from across the literature.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?



Surgery will be carried out according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017). In addition references such as A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes by Diehl et al. 2001, resources from NC3Rs (National Center for the Replacement, Refinement, and Reduction of Animals in Research, <https://nc3rs.org.uk/3rs-resources>), P. Flecknell (2015) Laboratory Animal Anaesthesia (Fourth Edition), S.L. Hoogstraten-Miller & P.A. Brown (2008), J. Bruce et al (2001), and other accepted literature as guided by the NVS.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The mouse development field is constantly evolving, and new breakthroughs are announced regularly in literature. Additionally, animal care staff are highly-trained and stay abreast of advancements in animal husbandry, bringing new techniques and procedures to scientists and assisting us with their implementation.

Explain the choice of species and the related life stages

Mice have been an excellent model system to study mammalian development for decades; they are prolific breeders, a close analog for human development, and there are many genetic mutants and fluorescent reporter lines available. For our proposals, there is no other animal model that could serve as a viable replacement or give us a closer understanding of human development (save for primate species). We use adult mice to produce embryos that are used in experiments, and embryos before the age of viability in order to study how organs and tissues are formed.





105. Motor circuitry in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Zebra fish	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 is the aim of this project?

Our aim is to understand how motor networks that coordinate movement develop and function and to understand how these circuits fail in diseases that affect motor systems.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Our work will improve our understanding of how neural networks in the brain and spinal cord interact to shape motor behaviour. Since motor-related circuitry in zebrafish is highly conserved with those of mammals we expect our findings to uncover general principles about how neural circuits interact to engender behavioural flexibility. In addition, our work will identify early, presymptomatic defects associated with neurological diseases such as amyotrophic lateral sclerosis and Parkinson's disease. Little is known of the defects that



occur during pre-clinical stages of these diseases and we hope that our work will help identify new therapeutic targets for slowing or preventing disease progression.

What outputs do you think you will see at the end of this project?

We expect to generate new knowledge relating to the fundamentals of motor control and the mechanisms underpinning neurodegenerative diseases of motor networks. Outputs generated by this project will comprise publication of peer-reviewed research articles, presentations at conferences and seminar sessions and public engagement at outreach events.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

By completion of this project, we expect our work to generate new knowledge on the basic principles of motor networks and the diseases that affect them. Outputs derived from our work will benefit a broad range of scientists within the academic community seeking to better understand the cellular and network mechanisms governing motor control and motor network diseases. Additionally, by completion of this project, outputs are expected to generate new insights and potential therapies for neurodegenerative disorders, thereby benefiting clinical researchers and medical professionals alike.

Members of the general public who are interested in aspects of motor control and disease of motor systems will also benefit from outputs generated during the course of this project as we will provide new insights into the mechanisms underpinning locomotion and early pathological process caused by degenerative diseases. In the longer term (5 to 10 years), we hope that new knowledge generated by this project will be essential for understanding and ultimately treating degenerative disorders affecting motor systems (such as ALS).

How will you maximise the outputs of your work?

We will maximise outputs generated by this project through i) publication of research findings in peer-reviewed internationally competitive journals. To maximise impact, we will ensure that work is published in Open Access format; ii) presentation of findings at scientific meetings and seminars; iii) attendance at outreach activities (such as REDACTED week and; iv) reporting of research findings on the laboratory website, which is accessible to the wider public.

Species and numbers of animals expected to be used

- Zebra fish: 4,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



Male and female adult zebrafish will be grouped or paired in a breeding tank. They will be left overnight and after spawning (which occurs at dawn), eggs will be collected and adults will be returned to their holding tanks to recover for at least 2 weeks before re-breeding.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The large majority of the GA breeding stock used for this project harbour genetically encoded fluorescent reporters that result in no discernible behavioural phenotype. We therefore do not anticipate that adverse effects will manifest in these animals. The neurodegenerative disease models we aim to use can develop muscle weakness and/or impaired motor performance at adult stages of life. However, our experience is that these phenotypes emerge in fish that are >2 years old. As animals used in this protocol will be euthanised by 21 months of age, we do not expect fish to develop harmful phenotypes during the course of the protocol. The breeding protocol is itself not expected to have any adverse effects on animals used in this project.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

100% of animals are expected to be in mild severity category.

What will happen to the animals at the end of the study?

- 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 study how vertebrates generate natural, flexible motor behaviours in response to sensory input. This work must be conducted using in vivo models: a holistic understanding of motor circuitry in health and disease requires models that are capable of generating naturally evoked behaviour. This can only be realistically achieved with in vivo preparations.

What was your strategy for searching for non-animal alternatives?

We have used the Frame.org website to screen for non-animal alternatives but none were available.

Why were they not suitable?

To understand motor network function in health and disease, intact in vivo models that are capable of generating natural bouts of motor activity in response to sensory input must be used. It is not possible to do this with mathematical models or human tissues.



Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

To maintain a breeding colony that can generate embryos on a daily basis, we will require around 100- 200 fish per genotype. We anticipate using around 10 GM lines. As fecundity decreases after 1.5-2 years, we anticipate replacing breeding stocks 3 times during the course of the project.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The number of animals requested represent the anticipated minimum to maintain a viable breeding colony for the duration of the project.

What other measures apart from good experimental design will you use to minimise numbers?

Wherever possible, progeny that are not used for experimental purposes will be reared for maintenance of our breeding colony. In addition, we will seek to share tissues from animals that are Schedule 1 killed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We require a stock of adult GA zebrafish for generation of embryos and larvae for experimental study. Breeding protocols are not considered harmful and the majority of GA lines used carry fluorescent reporter genes that do not cause pain, suffering or harm. GA lines that harbour disease causing genes are not expected to show clinical signs until >2 years. To ensure that clinical signs do not occur, these fish will be euthanised before 21 months of age using a Schedule 1 method.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

To study motor network function, in vivo vertebrate models must be used. Zebrafish offer an ethically- oriented alternative to mammals as the early stage fish that are not protected



under ASPA can be used for these purposes. We are required to use adult, breeding-age fish during this project so that embryos and larvae can be generated for study.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Health checks of our fish stocks are conducted twice a day. Fish displaying clinical signs are removed from the colony and killed via a Schedule 1 method. To maximise the success of breeding procedures and reduce metabolic and physical stress, fish will be cycled for breeding with individuals mated no more than every 2 weeks.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will adhere to the ARRIVE guidelines when designing, conducting and publishing research arising from this project.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We subscribe to the NC3Rs newsletter and will liaise regularly with our NC3Rs Regional Programme Manager to keep up to date with recent advances in the 3Rs. In addition, advances in the 3Rs will be monitored through attendance at conferences and symposia.

Explain the choice of species and the related life stages

Zebrafish are an outstanding model for the study of vertebrate motor systems because their motor networks are functionally and anatomically similar to our own. Moreover, their progeny are tractable to a range of powerful in vivo molecular, imaging and physiological methods. Therefore, we can use these early stage fish as an ethically-oriented alternative to mammalian models.

Adult zebrafish will be used as we require breeding age stocks to generate embryos and larvae for experimental study.





106. Mouse models of calcium, bone and endocrine 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

No answer provided

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The aim of this project is to understand the causes of disorders of calcium and bone metabolism such as osteoporosis, rickets, seizures (epilepsy), kidney stones, many cancers involving different organs, cataracts and diabetes. Understanding of the underlying biology of these disorders will also help with the development of new drugs, which is hampered by our current lack of knowledge of the abnormal physiological processes associated with these 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?



Our research studies focus on identifying the genetic causes of complex disorders of calcium balance, bone metabolism, kidney failure and tumours of endocrine glands (e.g. pituitary, parathyroid, pancreas, adrenal, ovaries, testes) with the ultimate goals of improving diagnosis and treatments for patients with these diseases. To achieve these goals, we need to study the abnormal physiological consequences of the genetic mutations identified in patients, and the only feasible way to accomplish this is to generate mutant mouse models for these diseases.

The disorders of calcium balance, which may be present as increased or decreased levels of calcium in the blood or urine compared to normal, result in many symptoms, including kidney stones, abdominal pain, pathogenic deposits of calcium salts in soft tissues, psychiatric disturbances, excessive urine production, cataracts, involuntary muscle contractions, and osteoporosis, as well as other disorders that do not involve calcium such as cardiovascular disease and diabetes. Furthermore, calcium disturbances may have multiple causes including: single gene disorders of calcium balance or bone metabolism; endocrine (parathyroid gland) tumours, or tumours of other organs (e.g. lungs and kidneys) producing parathyroid hormone related compounds; kidney failure; or decreased parathyroid hormone secretion which can lead to lower levels of calcium in the blood. Many of these disorders are relatively common and place a heavy burden on health care systems. For example: osteoporosis affects ~3 million people in the UK and leads to increased risk of fractures; kidney stones affect ~8% of the population by the age of 60 and frequently reoccur; parathyroid gland tumours have an annual incidence of 1-3 per 1,000 individuals; and changes in the development of embryonic tissue that give rise to structures within the head and neck, from which the parathyroid glands usually develop, have an incidence of 1 per 4,000 live births. There is an unmet clinical need for many of these disorders, either due to lack of current treatments, or imperfect treatments. For example, the only current interventions for kidney stones are dietary, and there are no drugs, resulting in ~10% recurring each year. Parathyroid tumours, or other cancers may be surgically removed; however, this is not always successful due to incomplete removal of the tumour and/or malignant growths away from the primary site, whilst conventional chemo- and radio-therapies are often ineffective for endocrine tumours due to their low growth rates. Current drug treatments for decreased parathyroid hormone production include calcium and vitamin D; however, these are associated with adverse effects including high calcium levels in the urine and kidney stones.

Our studies will help to understand the genetic causes, biochemical interactions, including hormone signalling, between different organs, in diseases where there are underlying disturbances of calcium. We propose to use mouse models, as mice maintain a high enough degree of similarity to humans in the regulation of calcium, and there are well developed techniques for modifying mouse genes, breeding and characterising the physical appearance and biochemical characteristics that result from these genes and their interaction with the environment. By using mouse models we will also be able to assess new and existing drugs, and dietary alterations, as well as providing new model systems in which novel therapies can be tested prior to their use in man. Thus, our studies are likely to increase knowledge of the disordered physiological processes associated with these diseases, as well as provide benefit to patients by improvements in diagnosis and treatments.

What outputs do you think you will see at the end of this project?

We anticipate that the outputs will include new information, publications and new treatments for patients as follows.



Full examination of the physical characteristics regulating calcium in blood and bone as well as non-calcium characteristics in novel REDACTED will be performed, which will increase understanding of disease causes (pathophysiology), and testing of therapies will increase knowledge of drug actions (pharmacology).

We are continuing our genetic sequencing studies of patients with disorders of calcium balance, bone metabolism, kidney function and endocrine gland tumours which may uncover new candidate genes and thus novel REDACTED based on mutations in these candidate genes may be generated. The biological advances from these studies will depend on the characteristics of the novel gene candidates, as illustrated by our previous studies of such human disorders, which have revealed the involvement of factors regulating: gene expression, biochemical reactions, signalling mechanisms within cells, movement of substances into or out of cells, and hormones.

Thus, these studies will reveal new biological information that will be published in scientific journals. Furthermore, these mutant mice will provide models for testing existing and novel drugs, as illustrated by our studies of mouse models with disorders of calcium balance, and endocrine pathways, in which we have evaluated the effects of drugs that: mimic the effect of calcium or hormones, compete with the effect of calcium, and regulate gene expression.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short-term, our studies will help to understand genetic causes, cellular pathways and the interactions between different organs, in diseases underlying endocrine and metabolic disturbances of calcium. For example, we will assess at least 7 different models of calcium homeostasis disorders due to mutations in e.g. *Gna11*, *Ap2s1*, *Casr*, *Nfix*, *Prlr* and *Men1*. Using these models we will be able to assess existing and new drugs and dietary alterations, as well as providing new model systems in which novel therapies can be tested prior to their use in man. Thus, research scientists and clinicians in different disciplines (e.g. endocrinology, metabolic medicine, genetics, paediatrics, nephrology, skeletal biology, molecular biology, physiology, biochemistry, pathology and pharmacology) will derive benefit from the knowledge.

In the long-term, pre-clinical studies will provide evidence for future clinical trials for researchers to test drugs that are novel, pre-existing or repurposed. Beneficiaries may therefore include patients, clinicians and the pharmacology industry. We have a number of existing collaborations with pharma companies to test drugs and facilitate any promising results into clinical trials.

How will you maximise the outputs of your work?

Results will be published in high impact peer-reviewed journals and presented at national and international conferences. Collaborations will be established with international groups, and novel mouse lines will also be made available through archives, for example European Mouse Mutant Archive EMMA.

Species and numbers of animals expected to be used

- Mice: 65000



Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

A typical experience for a mouse would comprise collecting blood by a needle from the tail daily for 5 days followed by analysis in a metabolic cage, followed by radiological imaging (e.g. by X-ray, DEXA or other scans) involving general anaesthesia to assess parameters of calcium, bone, kidney, and tumour metabolites followed by culling and removal of blood (a terminal bleed).

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Adverse effects specific to mouse models related to disorders of calcium homeostasis, bone metabolism, renal failure and endocrine tumours include:

Polyuria, polydipsia, abdominal pain, muscle weakness, fatigue and cardiac arrhythmias due to hypercalcaemia (~1-5%)

Tetany, muscle cramps, and seizures due to hypocalcaemia (<1%)

Skeletal abnormalities impinging on the ability to move around the cage and/or consume solid food due to disorders of bone metabolism (~5%)

Disturbances in balance and/or walking in a circular pattern due to an advanced pituitary tumour (<0.5% as they usually develop after the age at which these mice will be bred)

Reduced mobility and/or feeding due to tumour effects (<2% as they usually develop after the age at which these mice will be bred)

Increased urination or drinking due to disturbances in glucose metabolism (~10%)

Weight loss and loss of condition due to metabolic disturbances (<5%)

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

The majority of mice under this licence will be under the breeding protocols in which adverse effects will be mild or subthreshold for approximately 80% of mice and moderate for 20% of mice. A subset of mice (approximately 20%) will undergo the phenotyping or testing of agents protocols in which the development of clinical signs is required and they will have moderate adverse effects.

What will happen to the animals at the end of the study?



- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Regulation of plasma calcium levels requires the interaction of the parathyroid glands, bone, gut and kidneys, via circulating hormones and vitamin D. In addition, calcium has different effects on different organs such as the pancreas. As this represents a whole body system, it is not possible to investigate and understand disease development in isolated cells, and instead whole live animals are required. In addition, drugs need to be tested in whole animals so that the responses of all the different organs can be studied, thereby revealing on-target (i.e. efficacy) and off-target (i.e. side or adverse consequence) effects.

What was your strategy for searching for non-animal alternatives?

We are striving to develop cell models that may be able to replace some mice in some preliminary studies. For example, we are trying to immortalise and grow cells taken from mouse organs in the laboratory, and these could be used to test whether new drugs might be effective, before they are tested in live mice.

Why were they not suitable?

Regulation of calcium levels requires the interaction of multiple organs, it is not possible to investigate and understand disease development in isolated cells but instead whole live animals are required.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals for each experiment are calculated based on effect size where known from previous studies or the literature using power calculations to determine the chance of detecting any variations between test groups. Where effects size cannot be pre-determined then either pilot studies will be performed to establish preliminary data that can be used in power calculations, or the resource equation of Mead will be employed to give an estimate of sample size based on the general rule that the error degrees of freedom for a given experiment should lie between 10 and 20 to maximise the chance of detecting a difference between tests and avoid wasting resources (i.e. mice) for increasingly small gains in detection power. The number of animals estimated for each protocol are based on scaling up the anticipated number of experiments planned (based on estimates from our experience over previous years performing similar research studies to address similar



questions and numbers of test agents), which are then combined to give the total number of mice for the project.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Animals are only bred as required to supply animals for experimental requirements. The number of animals required for the experimental studies is determined by power calculations to achieve an effect size. The group is in contact with statisticians who offer advice on statistical requirements. Where possible randomisation and blinding will be used.

What other measures apart from good experimental design will you use to minimise numbers?

In all our mouse work, we use statistical analysis to ensure that the minimum number of mice are bred for the study, and that we use only the number of mice that are required to produce meaningful and useful results in order to answer the experimental questions. We are able to study the effects of drugs in multiple organs within an individual animal, for example in mice which develop tumours in more than one organ. Similarly, we can image the same mouse several times to study the development of organs or tumours, rather than using several mice once. We are also trying to establish cells from mice that will grow long-term in the laboratory. These could be used to replace some mice for the early stages of testing of new drugs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We use animal models of endocrine and metabolic disorders affecting calcium homeostasis, the skeleton and kidney functions as these are often interlinked in relevant human diseases, which include: familial hypercalcaemic hypocalciuria (FHH), autosomal dominant hypocalcaemia (ADH), endocrine tumour development, Marshall Smith syndrome and renal failure. The models we use are representative of disease in human patients and are closely monitored for signs of suffering when they are culled.

We are using well established analysis methods that have been developed by experts in mouse husbandry. We are using LASA guidelines for asepsis techniques such as injections.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?



Only mice will be used in our studies, and have been chosen as they represent the lowest mammalian species which allow the necessary genetic manipulations and display sufficient similarity to human organs and physiology.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We have developed expertise and experience in mouse welfare, and have refined our tests to ensure that the highest quality data is generated for the least welfare cost.

We are keen to minimise severity and increase the welfare of these animals. To ensure this, we will use non-invasive tests that only cause temporary discomfort where possible. For administration of drugs, a small pilot study will be undertaken for new drugs, with increased cage observations and welfare checks to ensure that the drug is safe. We also aim to use long acting drugs where possible to reduce the frequency of dosing. During every test, mice are closely observed and anaesthetics or analgesics used when appropriate.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We conform to the ARRIVE guidelines on animal studies.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We attend termly gold standard animal welfare meetings at which a REDACTED representative of the 3Rs gives regular updates. We also consult the 3Rs websites for techniques and alternative models. We regular perform literature searches to keep up to date with advances in animal welfare.

Explain the choice of species and the related life stages

The level of calcium is tightly regulated due to its roles in multiple biological processes, and disturbances of this cause many common diseases, including kidney stones, osteoporosis, many cancers including different organs, rickets, seizures (epilepsy), cataracts and diabetes. Calcium disturbances may have many underlying causes including defects in the calcium regulatory processes, hormone disturbances, kidney failure, and tumours, and many of these are not well treated by current drugs. Development of new drugs is hampered by our lack of knowledge of the underlying biology of these diseases, and we aim to understand this in more detail to aid the development of new and improved drugs.

Our studies will help to understand genetic causes, cellular pathways and the interactions, including hormone signalling, between different organs, in diseases underlying endocrine and metabolic disturbances of calcium. We will also be able to assess existing and new drugs and dietary alterations, and will provide new model systems in which novel therapies can be tested prior to their use in man.

We will use mouse models, as mice provide rapid and efficient breeding whilst maintaining a high enough degree of similarity to humans. We expect to use approximately 65,000 mice over the five years duration of the licence.



The maximum severity of this licence is expected to be moderate. The adverse effects experienced by any mouse will be monitored closely. The adverse effects of high calcium in the blood include increased drinking and urination, abdominal pain and muscle weakness, and of low calcium in the blood are muscle cramps and seizures (rare). Some of the mice may develop skeletal abnormalities, which may reduce their ability to walk normally. Some of the mice may develop tumours, which normally do not cause noticeable symptoms, but may occasionally interfere with normal movement. In order to study these human diseases, we have to allow them to develop in the mice; however, we will take steps wherever possible to reduce the adverse effects. All of the mice will be humanely culled at the end of the procedure.





107. Mouse models of mucosal 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

No answer provided

Animal types	Life stages
Mice	adult, aged, 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 is the aim of this project?

We aim to discover ways in which the immune system maintains health through training by microbes. Particularly focusing on the role of the microflora and related cell signalling pathways.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 has been a rise in incidences of cancer, inflammatory bowel disease, allergies and metabolic diseases over the last half century. Mounting evidence suggests that our diet, along with our microflora that live within us, is instrumental in training our immune systems, which when not functioning properly can lead to an increased risk in all many diseases.

What outputs do you think you will see at the end of this project?



This work will provide new insight into how genetic and environmental risk factors increase the risk of developing chronic diseases such as cancer, inflammatory bowel disease and obesity. The information generated will also give insight into how the microbiome may be manipulated, through probiotics, antibiotics and/or diet, useful in the treatment of these disorders. The outputs will comprise of publications in scientific journals, as well as in the lay press, and will hopefully help to inform the public on how to maintain a healthy lifestyle.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

A major benefit of this work will involve the dissemination of these novel insights not only to the wider scientific community, but also the public, to improve societal knowledge of how to prevent these diseases. Dissemination will involve the publication of scientific papers and engagement with The REDACTED Press Office to report the findings of our research in the media (articles and interviews) as well as presenting our work at public engagement events.

How will you maximise the outputs of your work?

We will engage with The REDACTED Press Office to report the findings of our research in the media (articles and interviews) as well as presenting our work at public engagement events. My group has active collaboration with researchers at national and international levels. We will actively collaborate with other researchers using complementary approaches to maximise the outputs generated in this project, including through the sharing of available experimental data and animal tissue. Members of my group and I attend national and international scientific conferences to present our work. I also teach Undergraduate and Postgraduate students at The REDACTED and regularly discuss my own research in teaching sessions as well as hosting 6-8 research project students per year in my laboratory.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will be bred in captivity to over or under-express certain genes that are known to play roles in various chronic diseases (such as cancer, inflammatory bowel disease, diabetes, etc). These genetic manipulations are known not to cause any noticeable harm to the animals in the normal, healthy state. These animals may then have their microbiome manipulated, for example through antibiotic administration, either in the drinking water or as an oral gavage to mimic human antibiotic usage. They also may be administered certain bacterial or fungal preparations that are known to be non-pathogenic components of a healthy microflora. The animals may also be treated with substances that will cause intestinal damage, such as inflammation or tumours. Induction of such damage is required



so that we may find out ways to treat the inflammation or tumours through using immunomodulatory agents and/or microbial preparations.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Cancer causes morbidity in humans only at advanced stages, once the tumour prevents an organ from working properly. None of our animals will be left long enough for this to happen, but if the tumour was starting to impact on physiological functions, we would expect the animal to lose >20% of its body weight, appear hunched or lose interest in eating/cleaning. Animals which receive substances to induce intestinal inflammation will be closely monitored for any signs of ill health. Sometimes an animal will develop diarrhea, a normal response to intestinal inflammation. Occasionally, if chronic, the diarrhea may lead to weight loss and/or rectal prolapse, hunching or inactivity.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

No animal will experience severe severity effects. It is not expected that any animal will experience moderate severity effects. If they do, it will only be very short term, as the animals are checked at least once daily. Any animal experiencing moderate severity adverse effects for longer than 24 hours will be subjected to schedule one and tissues harvested for analysis. At worst, 50% of animals on protocol 3 may experience some mild adverse effect, such as diarrhea and/or weight loss and 5% may experience moderate severity effects, such as weight loss over 20% or hunching. This is a 'normal' response to intestinal inflammation (such as when humans are infected with a gastrointestinal infection) and animals will be monitored closely for sustained or overt weight loss >20% body weight or hunching which are indicators of suffering.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 physiologically relevant, the study of host:microbe interactions requires the use of animal models, as currently in vitro intestinal models are insufficient to replicate certain critical functions, such as how the intestine responds to infection and how cancer cells evade the immune system. The mouse is chosen because it is a mammalian model, transgenic animals specific to the gene of interest are available and it is possible to manipulate the microbiome. Making mice the ideal model to assess the interaction of genetic vs. environmental factors in disease.

What was your strategy for searching for non-animal alternatives?



Our lab has extensively studied epithelial responses to microbial ligands, including LPS REDACTED secretory protein *in vitro*. We have also conducted preliminary *in vivo* studies using a *Drosophila* model of epithelial repair and we use simultaneous *in vitro* and *ex-vivo* human tissue models to inform our animal studies.

Why were they not suitable?

Whilst informative and providing us with preliminary data to support our *in vivo* aim, and narrowing down our lines of enquiry, we are limited when translating results from *in vitro*, non-mammalian and *ex- vivo* human systems to physiologically relevant pathologies and therefore other models support and reduce, but cannot entirely replace animal work. Certain epithelial functions, such as differentiation into multiple cell lineages, symmetric and non-symmetric cell division and the influence of non-epithelial cell types cannot be tested in *in vitro* models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on previous experiments and breeding strategies, approximately 1000 animals will be bred per year. This is accounting for animals that are genetically modified and wild-type controls, so 5000 animals total are estimated to be required. Approximately 10% of the mice used (about 500) may be purchased/transferred from approved suppliers and/or licenced institutions.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The proposed experiments and methods of analysis of the results have considered and will be discussed with an independent statistician at the The REDACTED. For most of the experiments sample sizes will be set using power analysis, generally using a significance level of 5%, a power of 80%, and a least practicable difference between groups of 25%. Otherwise, we will use the least number of animals to provide an adequate description, generally on the basis of previous experience. Post-hoc power analysis will be utilised during the course of these studies to update our knowledge of appropriate group sizes when the data becomes available.

What other measures apart from good experimental design will you use to minimise numbers?

Breeding of genetically modified mice can involve relatively large numbers of animals. The transgenic mouse breeding strategy aims to generate as few 'unusable' mice as possible. The numbers used will be minimised by breeding separate transgenic/knockout and wild-type lines wherever possible. As breeding will generate both, male and female mice will be used for all procedures and analysis will account for potential sexually dimorphic



responses. We will also make the data and tissues generated from these experiments available to collaborators both at The REDACTED and other institutions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice will be bred in captivity to over or under-express certain genes that are known to play roles in various chronic diseases (such as cancer, inflammatory bowel disease, diabetes, etc). These genetic manipulations are known not to cause any noticeable harm to the mice in the normal, healthy state. These mice may then have their microbiome manipulated, either through antibiotic administration, either in the drinking water, or as an oral gavage to mimic human antibiotic usage. They also may be administered certain bacterial or fungal preparations that are known to be non-disease causing components of a healthy microflora. The mice may also be treated with substances that will cause intestinal damage, such as inflammation or tumours. Induction of such damage is required so that we may find out ways to treat the inflammation or tumours through using immunomodulatory agents. Mice which have had tumours induced in them will be aged to no more than 12 months. Cancer causes morbidity in humans only at advanced stages, once the tumour prevents an organ from working properly. None of our animals will be left long enough for this to happen, but if it did, we would expect the animal to lose >20% of its body weight, appear hunched or lose interest in eating/cleaning. If any of these indicators of suffering are detected, the mouse will be humanely killed. Animals which receive substances to induce intestinal inflammation will be closely monitored for any signs of ill health.

Sometimes an animal will develop diarrhea, a normal response to intestinal inflammation. Occasionally, if chronic, the diarrhea may lead to weight loss and/or rectal prolapse, hunching or inactivity. Again, if this happens the animals will be humanely killed within 24 hours.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

As we are assessing the complex interaction of different cell types that have translational relevance to the chronic diseases experienced by human, we can not use species that are less sentient. Due to the chronic nature of diseases such as cancer, obesity and inflammatory bowel disease, we can not use animals at more immature life stages. Terminal anaesthesia would not be appropriate as the microflora changes, tumour development or intestinal lesions take time (weeks or months) to develop.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?



The most likely adverse effects on these protocols are weight loss and/or diarrhea. Weight will be monitored at minimum weekly (daily on protocol 3) and appearance observed daily at minimum. If any indicators of pain are observed, such as hunching, gurning or piloerection (caused by lack of self-grooming) the animals will be closely observed for a period of 24 hours, if no clear improvement is observed within 24 hours (i.e. lack of indicators of pain), the animals will be schedule 1 immediately.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow best practice guidance as provided by the nC3Rs to ensure that experiments are conducted in the most refined way, including guidance provided as part of the Experimental Design resources (<https://www.nc3rs.org.uk/experimental-design>) and by reporting our experimental data in accordance with the ARRIVE guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>). For pain assessment we refer to the nC3Rs mouse grimace scales.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We regularly monitor the NC3Rs website (<https://www.nc3rs.org.uk/>) for any news regarding advances in animal welfare and for any relevant events being held. We will also regularly attend scientific meetings with a strong 3Rs focus, including meetings held by the British Society for Immunology. We will also share best 3Rs practice with REDACTED colleagues through participation in the Universities Animal Welfare and Ethics Review Board (AWERB).

Explain the choice of species and the related life stages

Mice are most commonly used as the mammalian model of choice to explore how genetic in combination with environmental factors influence disease development. Gene expression can be manipulated in these animals to allow scientists to define how specific genes work to prevent (or cause) diseases. Advanced methods involving genetic manipulation in certain cell types, rather than all the cells in the animal, allow us to define relative contributions of certain cell types and make manipulations more relevant to human conditions (i.e. translational). Mice are also the ideal model to study the relative contribution of microflora as they can be kept clean and their flora can be removed (i.e. germ free). Much of the research that has led to what we know about the role of microflora in diseases, such as cancer, has been carried out in mice.





108. Mucosal Immunisation to Covid-19

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

To evaluate the use of bacterial spores as a vaccine delivery system focusing initially on the current Covid-19 pandemic.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 approach proposed has utility for the first stages in the development of mucosal vaccines. Mucosal vaccination is challenging and bacterial spores are attractive for this purpose. The current Covid-19 pandemic emphasises the need for mucosal vaccines which may be more appropriate for a disease of the upper respiratory tract.

What outputs do you think you will see at the end of this project?

The primary outcome of this work will be validation of the spore platform for delivery of immunogens to the mucosa and the production of mucosal immunity. This would lead to



downstream challenge experiments and ultimately a major step forward for the development of human vaccines for which no vaccines currently exist.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short term this work will evaluate and validate mucosal vaccine candidates that will be taken forward to challenge studies. This is an important first step in the program of pre-clinical events leading to human studies and new vaccines. In the current period Covid-19 is a global priority and so our efforts will be focused on validating the spore vaccine for early and rapid evaluation for a new mucosal Covid- 19 vaccine.

How will you maximise the outputs of your work?

REDACTED would like to publicise its results and raise awareness. This would occur through publications, conferences and participation with advocacy groups, learned societies, special interest groups and public health agencies

Species and numbers of animals expected to be used

- Mice: 2,450

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will be housed in appropriate groups and administered an immunogen by a mucosal route. Control groups will also be run in parallel for example, animals receiving an appropriate buffer (naive) or non-recombinant spores ('sham group') by the same delivery route. In addition, animals may also be immunised using a parenteral route to determine the immunogenicity of the antigen expressed on spores. This serves as an important control for ensuring the antigen is immunogenic.

During this procedure animals will be periodically sampled for analysis of antibody responses, this being via tail bleeds. After the study end animals will either killed using a schedule 1 procedure or taken forward to a challenge experiment (but out of the scope of this project licence).

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

No impact or adverse effects are expected from the immunisation and sampling procedures described here.

Expected severity categories and the proportion of animals in each category, per species.



What are the expected severities and the proportion of animals in each category (per species)?

For the majority of animals the severity is mild but for those under an AB procedure a proportion (<5%) will be Moderate.

What will happen to the animals at the end of the study?

- 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?

With current scientific product development of a human vaccine with either prophylactic or therapeutic properties it is necessary to first demonstrate that the prototype vaccine is: a) immunogenic and capable of producing a mucosal or systemic immune response, and b) immunised animals are protected from challenge with the live pathogen. These are the two most important criteria for progressing with a vaccination approach and must be addressed fully before any consideration of entering human clinical trials. We are not aware of any human vaccine that has entered clinical evaluation without the completion of pre-clinical studies in animals.

What was your strategy for searching for non-animal alternatives?

Currently, there are no non-animal alternatives available. It is worth emphasising that the decision to enter animal testing is itself determined by a number of in vitro tests that taken together support evaluation in animals. While in vitro tests are not confirmatory of efficacy they play an important role in the decision to evaluate in animals and the likelihood of success.

Why were they not suitable?

A disease caused by a microorganism (virus or bacterium) is nearly always multi-factorial, that is, the manifestations of disease result from a number of independent events. For example, for a virus this would include the impact of a multitude of cytokines produced in the host and for bacteria the production of toxins, attachment to a substrate, induction of innate immunity etc. Most of these events can be assessed individually using in vitro tests and taken together provide an indication that a test product may work but only using an animal model can this be categorically defined as positive or negative.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise



numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Previous studies will have used power calculations to determine group sizes and the validity of these calculations demonstrated from previous studies. As a rule, numbers of animals used per group should be sufficient to enable statistical significance between groups. Since the studies contained here have the same primary objectives we can use the same group sizes. If the primary objective will be different a new power calculation will be performed to determine animal numbers.

A typical study in mice would be 50-70 animals . For 5-7 studies per year for mice with overage this is equivalent to 3,000 mice.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

In the long-term animal numbers are reduced by careful project design reducing the need for further experiments. A core tenant of the design of animal experiments is the correct use of statistics (for interpretation of significance) and power calculations (to determine group size). The level of variability in some primary endpoints necessitates larger numbers to achieve statistically and biologically meaningful data. The most appropriate statistical tests Mann-Whitney (or the students t-test) for significance and ANOVA for analysis of variance between groups.

What other measures apart from good experimental design will you use to minimise numbers?

In our experience for studies are to be undertaken where large number of animals are to be used then small pilot studies using 2-3 animals/group are preferable. This is particularly important where a challenge dose must be determined or a dosing regimen established. Although statistical significance is not determined, in the long term, this avoids a negative outcome involving large numbers of animals.

Additional factors are to ensure the use of the same animal supplier (where possible) and the use of similar aged 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice will be used to evaluate mucosal immunity. This is achieved by mucosal delivery of the immunogen in parallel with control groups consisting of the immunogen alone administered by either a parenteral route (injection) or by a mucosal route if necessary.



The least invasive route of substance administration and using flexible gavage needles will be used where possible. Negative control groups (baseline groups) will be minimised whenever statistically feasible.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice are used since these are the lowest vertebrate group that can be used for evaluation of in vivo immunity.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Improvements in any process can always be made and we are receptive to any suggestions as well as our own assessments. We are keen to consider and if possible implement non-aversive handling of animals as well as practices for enrichment of quality of life for animals housed under solitary conditions. We will implement these where possible using only suitably trained staff and discussion with the REDACTED unit and NACWO representative as necessary.

To minimise discomfort of repeated procedures such as anaesthesia, we will combine treatments under a single anaesthetic event wherever possible. The anaesthesia will preferably entail the use of inhalation agents whenever possible. Least invasive route of substance administration and needle gauge will be used where possible. Negative control groups (baseline groups) will be minimised whenever statistically feasible.

Expertise at the Designated Site further enhances animal welfare, by providing close collaboration with dedicated animal care staff and veterinary consultants, and ready access to highly skilled advice.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

NC3R guidelines. We will follow the NC3Rs guidelines on the "Responsibility in the use of animals in bioscience research" and consult all the relevant references listed therein.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

By interaction with the Head of Animal unit at REDACTED and the NACWO Representative. Continuous monitoring of publications and the NC3Rs website for new and alternative models that could be implemented as part of this project and relevant information is circulated by AWERB. Whenever possible we will implement these refinements into our studies.

Explain the choice of species and the related life stages

Mice will be used to evaluate the ability of vaccines. Adult mice are routinely used for evaluation of immune responses by the scientific community. Mice can also be used for challenge experiments. Although out of the scope of this project licence vaccinated



animals could be evaluated subsequently in challenge experiments against the relevant pathogen. For example, immunised mice can be assessed for protection to influenza A.



109. Nanomedicine design and development

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - 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

No answer provided

Animal types	Life stages
Mice	adult
Rats	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

We want to find out how putting drugs into tiny particles improves their activity as 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?

Medicines must be discovered by first discovering the active ingredient and then once an active ingredient is identified it must be developed into a medicine (e.g. a tablet, injection, eye drop or suppository). Sometimes the nature of the active ingredient makes it hard to



make it into a medicine because it is fragile and easily destroyed when taken or because it cannot get inside the brain to treat brain diseases or it cannot get to the retina to treat diseases of blindness. Sometimes cancer drug active ingredients are hard to develop into medicines as they need to get in large quantity to the cancer itself and not to anywhere else. This is because when cancer drug active ingredients end up in healthy tissue, this causes side effects.

Putting medicines into tiny particles made of other materials (e.g. fats, carbohydrates and proteins) protects the active ingredients from destruction and helps these active ingredients get to where they need to go in the body. The tiny particles in question are really really small and are less than 1/100th of the width of a human hair in diameter.

We wish to study how these tiny particles work and how they shuttle drugs around the body so that ultimately we can design particle-based medicines to treat various conditions, e.g. brain diseases, diseases that cause blindness and cancers.

We have already used our knowledge of these tiny particles to create a pain medicine candidate and licensed this to a United States (US) company, for further development. This company will test this medicine in humans and see if it can replace the opioid pain killers (e.g. morphine and fentanyl) used currently. 17,000 people die every year from using opioids to control their painful conditions in the US and so a safer replacement will be welcome and extremely timely.

Furthermore we have used these tiny particles to create a dry-eye medicine which has been licensed to a US company and this company will again test this medicine in humans and see if it can replace the current approved medicine. The current leading dry eye medicine takes six months to show an effect. The licensing company believes that the medicine that we created could make patients respond faster.

Our success in the past, as described above, means that in the future, as well as benefiting patients, the work that we are doing is very likely to help the economy by building new companies, where relevant. Furthermore as the REDACTED owns the intellectual property that we generate from REDACTED funded projects, the REDACTED will reap benefits from the sale of such companies or the licensing of our discoveries.

What outputs do you think you will see at the end of this project?

At the end of our project, we expect to have learnt more about the way these tiny particles work in the body. If the way that these tiny particles work, as medicines, is better understood we will be able to design new medicines to treat brain disorders, diseases of blindness and some cancers. We will publish our work in scientific journals to allow other scientists to learn from our work and we may even develop some new medicines, if the data is promising. We have developed medicine candidates that will undergo clinical testing by others (e.g. the cyclosporine eye drop formulation and enkephalin formulation mentioned above) using these tiny particles in the past.

Industrial manufacture of these nanomedicines will have a limited environmental impact as we are currently using a waste product (chitin) to prepare our nanoparticles. However there will of course be an environmental impact as with all industrial manufacture (e.g. production of waste materials and energy usage).



What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short term (2 - 5 years), our work will benefit other scientists as they will learn from our published findings. Our work will also benefit the scientists working on our programmes as well as ourselves as we will all learn something new about the way these tiny particles work as medicines.

In the medium term (5 - 10 years), our work will benefit the economy if new medicines are developed or are in the process of being developed as these discoveries will lead to money coming to the REDACTED when these discoveries are out-licensed to third parties.

Ultimately in the long term (10 - 15 years), if new medicines are developed and approved for sale, our work will benefit patients who suffer from brain diseases such as chronic pain or dementia or patients who are at risk of blindness. Finally our work may even benefit cancer patients.

How will you maximise the outputs of your work?

We will work with other scientists and engineers to make sure that we fully understand what these tiny particles are capable of as medicines. For example, we already collaborate with physicists as well as with eye doctors. These collaborators help us to understand how the formulations that we are developing may be used (e.g. clinicians) and help us understand the mechanisms of action of various potential medicines (e.g. physicists working with us on gold nanoparticles).

We will make sure that any findings that may be used to make new and improved medicines are taken forward either by establishing a new company or by licensing our findings to an established company so that the medicines may be developed.

We will publish all of our work, even when the results of the experiments show that the tiny particles are unable to shuttle active ingredients to particular areas. This will help scientists understand these particles better and inform others on which avenues to pursue within the discipline.

Species and numbers of animals expected to be used

- Mice: 6000
- Rats: 4000
- Rabbits: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The animal will be bought from a reputable company and then transported to our animal house where it will live for 7 days with food and water supplied in order for the animal to



get used to the new environment. Environmental enrichment will be in the cage so that the animal is not bored.

On Day one of the experimental phase of the project, the animal will be dosed some of our tiny particles and the dose will be such that it is unlikely to cause harm as the particles would have been tested in cells first. The animals may be dosed more than once and the dose volumes will be within the acceptable limits. Animals may be dosed with solids or liquids and via different routes. The animals will be killed after a suitable period of time and their organs and blood taken for us to analyse.

Sometimes blood will be taken repeatedly from one animal over a one or two day period but this will be no more than 10% of the total blood volume over a 24 hour period and no more than 15% of the total blood volume over a 48 hour period. The animal will be humanely killed at the end of this study.

Some animals (mice and rats only) will be disease models (e.g. a pain model to test if the analgesics are working) and so they may be subjected to moderate pain in order to see if the tiny particles reduce this pain. A typical pain experiment may involve the animal having its tail dipped into hot water of 50 degrees centigrade. 50 degrees centigrade is just hot enough for a human to immediately withdraw their hand but not hot enough to cause scalding. The animal will be able to immediately withdraw its tail from the water if it feels painful and will keep its tail in the hot water longer if the tiny particles are giving the animal pain relief. Another typical pain model is a nerve damaged model. In all cases we will measure the pain relief felt by the animal. The animal will be humanely killed at the end of this study.

Only healthy rabbits will be used for our studies.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We intend to obtain results which will tell us that the tiny particles are working in the animals either to bring about relief from a disease state or that they go to areas in the body where we want the particles to go.

Some animals may suffer weight loss of more than 15%, moderate pain, show signs of discomfort by remaining hunched over or make unusual sounds. We do not expect more than 5% of our animals to show these effects.

Some animals may suffer from the anaesthetics that we give them and have an unexpected effect that shuts down their organs. We do not expect more than 2% of our animals to show these effects.

Common effects like bruising after we have withdrawn blood may affect about 10% of the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?



We expect 90% of mice to have mild effects and 10% of animals to have moderate effects.

We expect 90% of rats to have mild effects and 10% of animals to have moderate effects.

We expect 95% of rabbits to have mild effects and 5% of animals to have moderate effects.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 tiny particles that we are testing are carried by the blood to the various organs and so we need to test them in the presence of flowing blood. This can only be done in live animals.

What was your strategy for searching for non-animal alternatives?

We considered the use of computer models and organs grown in laboratories.

Why were they not suitable?

The computer models are not sophisticated enough to really mimic blood flowing in a mammal and to mimic all the cells and organs in a typical mammal.

The organs grown in laboratories do not have a well developed blood stream that we can use and test how the tiny particles shuttle medicines around these laboratory grown organs.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers below are the upper limits for our animal numbers.

We will always first conduct a pilot experiment to measure the size of the response and by how much it varies and this will be used to decide how many animals are needed in each group. The animal numbers in each experiment, given below, come from experiments that have been conducted in our laboratory previously. Group sizes have been determined using statistical methods



Mice - For group sizes of at least 5 animals and with a test and one control group and sampling at 8 time points, we estimate that each experiment will need about 100 animals (including the pilot studies) and we will conduct about 12 experiments per year and hence will need 6000 over 5 years.

Rats - For group sizes of at least 5 animals and with a test and one control group and terminal organ sampling at 6 time points, we estimate that each experiment will need about 80 animals (including pilot studies) and we will conduct about 10 experiments per year and so we will need 4000 over 5 years.

Rabbits - For group sizes of at least 5 animals and with a test and one control group and terminal organ sampling at 4 time points, We estimate that each experiment will need 50 rabbits and we will conduct about 2 experiments per year and so we will need 500 rabbits over 5 years.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We need each experiment to yield meaningful data that we are able to use. This will limit the overall number of animals used, as experiments then do not need to be duplicated.

We used published guidelines to design our experiments (e.g. the PREPARE guidelines and the NC3Rs' Experimental Design Assistant).

We have used power calculations to make sure that our experiments contain enough animals so that we are able to detect any differences in our samples.

What other measures apart from good experimental design will you use to minimise numbers?

We will carry out pilot experiments to see how big our signal is and by how much it varies. We will then use this information to calculate the best group size for our experiments that will give us reliable 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use healthy mice, rats and rabbits to study where the tiny particles go in the animal. These models are best as there will be no presence of disease in these models and we will make sure that the dose that we select for our main studies have been shown not to cause harm in a pilot study of a few animals.



When we want to measure whether the particles work in the animal as potential medicines, we may use disease models, but these disease models will be such that the disease/condition does not cause severe harm.

We plan to use pain models but have chosen pain models that do not cause excessive suffering. Pain models include: dipping the animal's tail into hot water of 50 degrees centigrade, standing the animal on a hot plate of 50 degrees centigrade, lightly damaging a nerve supplying the hind limbs or injecting the animal with a compound that causes paw inflammation. All of these pain models cause the least pain and suffering for the animals but allow us to know if the medicines that we are developing for painful conditions actually work.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The tiny particles need to be tested in mammals with a blood flow that is comparable to that found in human beings.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We will minimise animal suffering by making sure that the tiny particles that we give the animals have first been tested on cells and will give them at a dose that theoretically will not kill the cells in the first instance. We will then take the dose up slowly observing for any harm along the way and stopping the experiment if we observe harm that is above our moderate limit.

We will observe all animals for a period after dosing to make sure that we can pick up any unexpected bad effects. In any event we will check our animals daily if we are doing longer experiments.

With any surgery or other use of anaesthetics we will monitor the animals after the procedures to make sure that we pick up any unexpected bad effects.

Apart from when specifically testing in a pain model. we will give the animals analgesics if we observe them to be in pain (e.g. after surgery) and will give the animals analgesics beforehand if we anticipate any procedures may cause pain (e.g. before surgery).

All surgery will be done under general anaesthesia.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will use the ARRIVE and PREPARE guidelines.

We will use the LASA Administration of Substances guidelines.

We will use the LASA Guiding Principles for Preparation for and Undertaking Aseptic Surgery

How will you ensure you continue to use the most refined methods during the lifetime of this project?



We will read the literature and monitor updates to the NC3R, PREPARE and ARRIVE guidelines. These are laboratory practice guides that tell you how to conduct animal experiments in a way that minimises animal harms.

Explain the choice of species and the related life stages

We need to study where the tiny particles go in the body and so we are using adult mice, rats and rabbits as these are the lowest vertebral life forms that are able to provide us with meaningful results.

We need to study how the medicines made from these tiny particles work and so we are using adult mice, adult rats and adult rabbits, as these are the lowest vertebral life forms that are able to provide us with meaningful results.





110. Natural killer cell therapy for viral infections

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

No answer provided

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 is the aim of this project?

The aim is to develop new ways of activating natural killer cells to combat viral infections, especially COVID-19.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

COVID-19 is currently causing a worldwide pandemic, with over 20,000 deaths in the UK alone.

Currently we are in the midst of an emergency with respect to this viral infection and it is critical to devise new methodologies to prevent spread of infection. We propose that one method would be to activate natural killer cells to limit infection in infected individuals. This approach is distinct from other strategies and based on our original fundamental science.

What outputs do you think you will see at the end of this project?



The main output is to find a new way to treat COVID-19. We will determine if the vaccine is safe in mice prior to human studies.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The main benefit will be the general public as the work forms the basis for a new treatment for COVID- 19. This may take up to 2-3 years. The academic community will also benefit through presentations at conferences and publications. In the short term (6 months to one year) the benefit will be to the academic community through presentations. Publications would be anticipated within 2 years.

How will you maximise the outputs of your work?

The works will be presented to the academic community at national and international meetings and also through the scientific literature to disseminate the findings. We will also disseminate the work locally through academic meetings and presentations. Significant findings can be disseminated through the REDACTED press office and also public engagement activities such as "Pint of Science". The work may have benefit to other RNA viral infections especially flaviviruses, which we can indicate through the academic press, such as through review articles and presentations.

Species and numbers of animals expected to be used

- Mice: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

In a typical experiment adult KIR-transgenic mice will receive two DNA vaccine injections into the thigh muscle. These injections will be given one week apart and injections will be into alternate thighs. The mice will be monitored for any adverse events related to the vaccine. They will have blood taken via the tail vein on one occasion. The mice will be killed humanely one week later, using a Schedule 1 method and a post-mortem examination of the internal organs performed.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Animals may experience local irritation to injections. This is usually mild and passes within 24 hours. We anticipate that by operating within the guidelines for blood drawing and with careful monitoring then animals are not expected to experience significant adverse effects related to these procedures.



Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

We anticipate the vast majority of animals will have severity scores of mild, and that only in rare case will they have a severity score of moderate.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 develop and test our vaccine strategy. The ultimate goal of this project licence is to devise a new way to treat human viral infections, especially COVID-19, but this an assessment of safety in animals before we can move to human studies.

What was your strategy for searching for non-animal alternatives?

We have gained as much information as possible from our previous in vitro experiments. However safety testing requires testing in an animal experiment before using in humans.

Why were they not suitable?

We have not identified a system that can faithfully recapitulate the response to a vaccine that targets natural killer cells. We have been unable to identify a suitable in silico or in vitro model for this work. In general these types of model systems do not recapitulate a whole animal experiment that is required prior to using a potential therapeutic in humans. This is because generating an immune response requires multiple steps and the interplay of many different immune cells in a co-ordinated fashion within a localised environment. This interplay cannot be readily or faithfully reproduced in tissue culture conditions to the standards required to inform a clinical trial. Furthermore, unanticipated toxicities cannot be readily identified in in vitro model systems

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?



We have estimated the number required based on the number of different experiments that we require to investigate safety of the vaccine, and perform the relevant controls, that will allow us to find unanticipated side effects related to the vaccine. In general we find that the vaccine is safe and well tolerated from studies in cancer immunotherapy. If we use 5 animals per grouping then this will allow for 20 different experimental conditions including control unvaccinated mice. We propose that this number will be sufficient for regulatory submission based on previous vaccine studies at our institution.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

In the design phase we have used inbred mice to reduce intra-group variability and allowing reduced mouse numbers for experiments. We have collected information from previous use of the vaccine in mice to help determine the likelihood of clinical effects related to the vaccine.

What other measures apart from good experimental design will you use to minimise numbers?

We have previously tested the vaccine in mice and not observed any specific toxicities. We therefore have made an assessment of the number required for these experiments and will use the minimum required to observe any clinical toxicities. We need to perform these studies to gain sufficient clinical data to submit for regulatory approval. We had not previously planned to use the data from our previous work for regulatory approval as they were designed as a scientific investigation of NK cell responses to cancer to assess immunogenicity rather than safety, so mice were subjected to the minimum monitoring required for the investigative purpose. We do not feel that the previous level of monitoring would be sufficient for regulatory approval.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use a genetically altered immunocompromised mice as our animal model (KIR-transgenic (KIR- Tg; KIR+/+Kb-/-Db-/-). This is the most relevant model as it is the only animal model available that expresses the human KIR gene KIR2DS2. Therefore it is the most relevant to the human situation and so by using this it is most refined for our purpose. By using the best available model this ensures that the fewest possible animals are required for our project.

We will use injection of DNA by the intramuscular route as this is the method that generates a known immune response and side effects are mild and transient. We will use venepuncture from a superficial tail vein as this is the least harmful method for taking a sample of peripheral blood. In general mice will be kept for only a week after vaccination and then killed to minimise the risk of experiencing any lasting harm.



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice are the least sentient mammal species with an immune system similar to humans. The KIR-Tg mouse represents the most relevant animal model for these studies as it is the only animal strain that expresses the human KIR2DS2 gene which we are targeting.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Environmental enrichment, good husbandry and frequent monitoring ensure high welfare standards. Few adverse effects are anticipated but, should any occur, rapid steps will be taken to ameliorate them or humanely kill humanely affected animals. All animals will be maintained by qualified and experienced animal technicians who are familiar with the models. Mice will be handled using non-aversive methods eg not picking up by the tail, but moved using a tunnel or a cupped hand.

Overall, we have found under our existing PPL that the severity of most experiments is 'mild'.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will use the NC3Rs as a resource for our animal studies and experimental design. We will use information available in the NC3Rs website (<https://www.nc3rs.org.uk/3rs-resources>). Guidelines contained there include "Responsibility in the use of animals in bioscience research" and the "ARRIVE" guidelines for reporting the use of research using animals.

We will also use information from Norecopa and will follow PREPARE guidelines (<https://norecopa.no/prepare>) for all animal experiments.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will stay informed through the regular meetings of the animal facility and the user groups which occur every 3 months. Additionally, we receive e-mails to update us about changes in policy or practice. We will also check on-line databases to identify any changes such as the NC3Rs, Altweb and Norecopa web pages. Any changes will be implemented directly through the experimental design, and if necessary through a project license amendment.

Explain the choice of species and the related life stages

We are using mice as these are the least sentient animal that we can perform these experiments on. As the vaccine targets a uniquely human gene (KIR2DS2) then a transgenic animal is required to test the safety of the vaccine. We are using adult mice as these are the most resilient life stage and allow the experiments to be standardised. In general they will be studied between weeks 12-16, well before age-related effects will be observed.





111. Neural Control of Reproduction

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Rats	neonate, juvenile, adult, pregnant
Mice	pregnant, neonate, adult, juvenile, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

To determine the neurophysiological mechanisms behind the timing of puberty and subsequent reproductive function in adulthood.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Consequences of altered pubertal development in both man and animals, early or delayed, is linked to a myriad of reproductive, sexual, behavioural, mental or other health issues with lifelong social, health and economic implications. Further knowledge is needed on the basic mechanisms underlying puberty control, not only to improve health and social outcomes in modern society but also to increase the potential for applied aspects of puberty control in the farming sector, leading to improvement of production, welfare and sustainability.

What outputs do you think you will see at the end of this project?



Outputs will include new information about the central control of the hypothalamic-pituitary-gonadal axis, with a focus on the mechanisms upstream of the GnRH pulse generator. This will encompass reproduction in adulthood as well as pubertal development under normal and pathophysiological conditions. The aim is to publish these findings in academic journals.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The primary, immediate benefit of this scheme of work will be to advance fundamental scientific knowledge about the central control of the hypothalamic-pituitary-gonadal axis, with a focus on the mechanisms upstream of the GnRH pulse generator. Our investigations include both reproduction in adulthood as well as pubertal development under normal and pathophysiological conditions. The aim is to publish our findings in academic journals. The information is likely to be of interest to physiologists and neuroendocrinologists with an interest in the mechanics of reproductive physiology or stress.

A more long-term secondary potential benefit relates to possible clinical application of our findings to reproductive dysfunction or stress-induced disorders observed in humans. Work in abnormal thermoregulation may have specific applications for cancer patients and postmenopausal women suffering extreme hot flashes. Our investigation of the regulation of the hypothalamic-pituitary-gonadal axis under stressed conditions may provide targets for the development of pharmaceuticals for the treatment of ovulatory dysfunction. The work relating to the central mechanisms involved in differential stress responses may also be of value in generating pharmacological treatments for stress-related disorders such as anxiety and depression.

How will you maximise the outputs of your work?

Our methods and results will be published in a variety of journals including some that are open-access so that this work reaches the wider scientific community. Results will also be presented at meetings as well as national and international conferences. We have collaborations with researchers from other institutions, including internationally, which ensures that this work reaches other fields as well as our own.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



A maximum of five general anaesthetics can be administered, although animals will typically undergo no more than two surgical procedures (and therefore only two anaesthetics will be required).

The typical mouse procedure involves injection of extremely small quantities of a light sensitive proteins into the brain, followed by implantation of a micro fibre-optic probe to allow for optogenetic stimulation and/or brain cannula for administration of pharmacological agents (none are expected to carry adverse effects). This procedure is often done at the same time as removal of the gonads. Animals will then undergo blood sampling. Some animals (30%) may be subject to environmental stressors to examine the impacts of stress on the reproductive axis. These are not expected to have persistent, long-term effects and animals will be taken care of accordingly on the off-chance the intended severity threshold is exceeded. The typical duration of the studies is 6 weeks and not more than 6 months. However, studies using animals exposed to early life stressors and followed into adulthood will typically last for 8 weeks, but not more than 6 months.

The typical rat procedure involves implantation of an intracerebral cannula, mainly for administration of pharmacological agents. Again, this may be done at the same time as gonadectomy. Rats will also be implanted with catheters for automated blood sampling. Certain animals (50%) may be subject to environmental stressors but these are not expected to have persistent effects. Typical duration of studies is 6 weeks and not more than 6 months.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The inevitable adverse effect of surgery is pain. Any animals undergoing surgical intervention of any kind will receive a mandatory pre-operative analgesic dose, and post-operative analgesia as required: animals will be individually assessed and monitored during the post-operative period, and given appropriate analgesia in accordance with any signs of pain they display. If, after 48 hours post-surgery they have not returned to normal (i.e. normal movement, well-groomed) and still show any of the following signs: hunched posture, piloerection, weight loss exceeding 15% compared with age matched controls, the Named Veterinary Surgeon will be consulted on their condition, or they will be humanely killed.

No adverse effects are anticipated when the animal is under general anaesthesia. However, it should be noted that possible lightening of the anaesthetic may occur; this is determined by a response to pinch, or by the eye blink reflex; additional anaesthetic is administered under such circumstances. Animals will be monitored for anaesthesia and recovery.

Possible adverse effects of developmental/early life stressors: for neonatal handling there is a possible increased likelihood of cannibalism; this is exceptionally rare. To minimise this risk gloves will be worn when handling the neonates and the scent of the home cage will be spread over the neonates before reintroducing the dam. During rearing, animals will be carefully monitored for appearance of any abnormalities. Animals showing any signs of ill health will be monitored closely in consultation with the Named Veterinary Surgeon, and if necessary killed by schedule one procedure. It is to be noted that no adverse effects of post-weaning social isolation on health or growth rates of the animals are expected.

Possible adverse effects of non-developmental stressors:-



A possible adverse effect of restraint stress is distress as evident by struggling which leads to injury. Animals will be observed continuously during restraint and removed from device if necessary.

A possible adverse effect of an acute immunological challenge, which is typically lipopolysaccharide (e.g. Escherichia coli) injected intravenously or intraperitoneally, is a transient fever and/or loss of appetite with weight loss. If adverse conditions remain for more than 12 hours the animal will be killed by a schedule one method. However, as the doses typically used are very low, it is extremely unlikely that illness caused by this immune challenge will be anything other than very transient (typically lasting 1-3 hours).

No adverse effects are expected nor have we observed them for the following stressors, i.e., insulin- induced hypoglycaemia, noise, predator odour, raised ambient temperature or cage switching.

Adverse effect of adrenalectomy is disturbance to electrolyte balance, which is minimized by salt replacement in the drinking water. No further adverse effects are expected from adrenalectomy, as adrenal hormones will also be replaced in the drinking water.

Adverse reaction to administration of drug (e.g. intracerebroventricularly, intravenously) is extremely rare. If this occurs the animal will be killed by schedule one method.

A possible adverse effect of implanting catheters, osmotic mini-pumps, transmitter devices, brain fluid or optic cannulae, or cranial screws for the attachment of tether-swivel assemblies which is minimized by using aseptic surgical techniques is infection at the wound site (rare). The animal will be treated with antibiotics on advice of the Named Veterinary Surgeon and the wound closely monitored. The experiment will be terminated and the animal killed by a schedule one method if no improvement. Wound breakdown following surgery (incidence very uncommon) is a possibility and re-suturing under general anaesthesia with recovery performed on one occasion only.

No adverse effects are anticipated with the use of the tether-swivel assemblies.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

The majority of animals will undergo surgical procedures (moderate severity) and approximately 25% may be exposure to stressors (mild to moderate severity). After recovery from surgical procedures, the majority of animals will under go blood sampling procedures to monitor luteinising hormone pulses in the blood as a measure the brain oscillator called the GnRH pulse generator that controls reproduction. This oscillator is exquisitely sensitive to stress so the animals are extensively handles and habituated in order to visualise the normal pattern of luteinising hormone pulses.

What will happen to the animals at the end of the study?

- Killed

Replacement



State what non-animal alternatives are available in this field, which alternatives you have 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 programme will use both in vitro techniques and animal procedures. The in vitro work will include examining biological activity in selected cell lines and tissue to elucidate signal transduction pathways and gene expression using molecular biological techniques. However, animal work is essential because the control of the GnRH pulse generator depends on integrated physiological systems.

Indeed, it is impossible to study the operation of the GnRH pulse generator by using cultured cells alone. For example, to measure the impact of stressors such as psychological/nutritional perturbations (e.g. restraint/food withdrawal, which are relevant to anxiety/anorectic models), requires a cognisant animal model. However, studies at the cellular level (e.g. signal transduction mechanisms or factors involved in neuropeptide receptor gene expression) give vital information that can be used in a complimentary manner to results obtained from these in vivo studies.

We also intend to use brain slices from animals killed by Schedule 1 methods where possible (cervical dislocation followed by decapitation and the use of an oscillating microtome to look at specific events, for example the expression of GABA/glutamate/kisspeptin during puberty).

What was your strategy for searching for non-animal alternatives?

Non-animal alternatives include examining biological activity in selected cell lines and tissue to elucidate signal transduction pathways and gene expression using molecular biological techniques. In silico techniques can be used to model GnRH pulse generator firing.

Why were they not suitable?

Although these methods will be integrated alongside animal work, the control of the GnRH pulse generator depends on integrated physiological systems and therefore requires use of a whole organism. Indeed, it is impossible to study the operation of the GnRH pulse generator by using cultured cells alone. To measure the impact of stressors such as psychological/nutritional perturbations (e.g. restraint/food withdrawal, which are relevant to anxiety/anorectic models), requires a cognisant animal model.

Examination of specific events such as the expression of GABA/glutamate/kisspeptin during puberty requires use of fresh tissue.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?



We have drawn on years of previous experience to ensure we use the minimum number of animals required. Statistical power analysis before initiating experiments will also be used to ensure that we only use the minimum number of animals required to produce valid statistical comparisons between the groups.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The design of individual experiments will generally involve factorial designs, to maximise the information gathered using the minimum number of animals. Most measures taken will be quantitative, in which case sample sizes will be set using a power analysis, using a significance level of 5% and a power level of 80%, with a least practicable difference between groups of 25%. This will generally result in group sizes of ~8 per treatment group, although in cases where very small and delicate structures in the brain are being targeted as cannulation or lesion sites, it is necessary to include more animals per group to ensure sufficient data can be collected if some of the cannulae/lesions are not on target. For design of complex experiments, advice of a statistician will be sought.

What other measures apart from good experimental design will you use to minimise numbers?

Use of factorial designs will maximise the information gathered using the minimum number of animals. We will also use in silico methods to mathematically model the interaction of these neuroendocrine pathways, this reduce the number of animals used in future experiments. Tissue from killed animals will be further utilised e.g. analysed to examine specific events such as the expression of GABA/glutamate/kisspeptin during puberty and the expression of stress related neuropeptides. This ensures a small number of animals are made use of as much as possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Rodents are the lowest vertebrate group which will allow reliable electrophysiological studies and in which the reproduction control systems have been well characterised. They are excellent for monitoring the activity of the GnRH pulse generator either electrophysiologically or via collection of serial blood samples for measurement of luteinizing hormone pulses.

Due to small overall size, a mouse model is may not be suitable for some of the work; it is still the most common option for genetic alteration studies. As rat strains with our required genetic manipulations become available, they will be used to give the improved range of measurements and interpretation that cannot be provided by mice.



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Rodents are the lowest vertebrate group which will allow reliable electrophysiological studies and in which the reproduction control systems have been well characterised. Investigating the control of the GnRH pulse generator depends on integrated physiological systems and therefore requires juvenile or adult animals.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals will be monitored throughout all experiments to minimise pain or discomfort as a result; animals deemed to be suffering will be removed via a Schedule 1 method. Pre-operative analgesia will be administered to all animals undergoing surgery, and no animals will undergo experimental procedures if displaying any signs of pain. Most surgical procedures or drugs we propose to administer have limited, if any, persistent adverse effects. Neurotoxic lesioning of brain nuclei may very occasionally have unpredictable side effects such as loss of mobility or appetite, but in such cases, the animals will immediately be killed humanely if showing signs of ill health, such as weight loss, piloerection, ungroomed or hunched appearance.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) guidelines as well as ensuring we keep up to date on practices in the current literature to ensure our experiments are conducted in the most refined way.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will keep up to date on new advances in the 3Rs through the NC3Rs newsletter as well as regularly checking the 3Rs website.

Explain the choice of species and the related life stages

Rodents (i.e. rats and mice) are the lowest vertebrate group which will allow reliable electrophysiological studies and in which the reproduction control systems have been well characterised. They are excellent for monitoring the activity of the GnRH pulse generator either electrophysiologically or via collection of serial blood samples for measurement of luteinizing hormone pulses.

Since we are examining the effect of early life stress on reproductive function during puberty and in later adulthood, neonatal and juvenile animals will be used as well as adults. We also have our own breeding protocol which therefore includes use of pregnant animals.





112. Neural mechanisms in health and disease, therapeutics 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

No answer provided

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The overall aim of this project, that is built upon our previous achievements and innovative findings, is to provide new insights into the fundamental primary molecular mechanisms that control the behaviour of nerve cells in high-order functions such as learning and memory, as well as motor activity and metabolism in health and disease. At the same time, we will also explore the potential of novel candidate molecules resulting from our work for their potential to prevent initial degenerative steps in disorders such as Huntington's or Alzheimer's 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?

Advancing knowledge into these mechanisms is paramount to new or improved treatments for neurodegenerative diseases such as Huntington's (HD) or Alzheimer's (AD), and metabolic diseases such as obesity, diabetes.



HD is a rare genetic disorder caused by a defect in a single gene, namely a poly-Q expansion in the Huntingtin protein (HTT). It typically develops in adult life and induces progressive degeneration of brain nerve cells, leading to motor, cognitive, and psychiatric abnormalities and ultimately death. It affects between 10.6–13.7 individuals per 100,000 in Western populations. This devastating disease course generates a considerable societal burden, impacting social functions, employment and health care provision, as well as family members and carers. The care burden rises rapidly in advanced stages of disease, and overall UK cost for HD patients are estimated at £195M/year. No effective HD treatments currently exist, and several clinical trials have failed.

AD is the most common type of neurodegenerative dementia, accounting for 60 to 80% of dementia cases. It affects more than 24 million people worldwide. The majority of cases manifest as a late-onset sporadic form. Although progress has been made about the molecular pathogenesis of AD, we still do not fully understand the aetiology of the sporadic form. The most desirable solution is to delay its onset or to prevent it from developing. Currently there is no cure, and treatments available can only delay/slow the symptoms but are not able to stop disease progression. Overall UK cost for dementia is around £26 billion a year.

Control of food intake and energy balance, which is essential for animal healthy life, is a primary target of investigation due to the fact that dysregulation of these leads to metabolic dysfunctions such as obesity, type 2 diabetes. An estimated £14 billion pounds is spent a year in the UK to treat these and their complications. However, it is not well-established which neurons in particular nuclei of the brain control particular physiological functions, which is paramount to design more effective treatments.

What outputs do you think you will see at the end of this project?

This work is expected to provide new fundamental knowledge and publications about the molecular mechanisms that control the behaviour of nerve cells particularly in high order functions such as learning and memory, as well as locomotor activity and cell metabolism. This will advance our understanding about how molecular processes malfunction in disease. Overall, we may identify pathways and factors involved in the initial stages of neurodegenerative processes which could lead to the discovery of new strategies with the potential to prevent disease onset and treating disorders like Alzheimer's or Huntington's. As for the metabolic studies we will further define basic molecular mechanisms and eventual new ways to design more effective treatments for disorders such as obesity.

We anticipate that these primary benefits will be realised in the short- to medium-term, i.e. within the timeframe of this licence. This programme of work builds upon the successful achievements of the previous project licence. All methods required to achieve the proposed objectives are well established and I have been successfully using most of these methods on my current project licence. The most critical mouse models are already in place and the team has highly-relevant expertise in these. Thus, it is highly likely that we will successfully realise the primary benefits of this programme of work. Moreover, discovery of novel potential drug targets with the discussed approach could lead to the opportunity to engage with industry for translational studies (long-term benefit). We have already discussed with industries the potential of some of these studies. They believe that a small molecule screen could be a viable approach once a particular target is more corroborated. Therefore, in the short term (by mid-PPL) we envision to have done more mechanistic studies of the above findings and medium-term (end of PPL and shortly after)



to have the characterization done in an appropriate model of disease, getting ready for an eventual small molecule screening in the long-term.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

These studies have a transforming potential for the human patients as there are no effective treatments for preventing or delaying this type of disease, or even to improve symptoms. The development of effective treatments would also have a positive impact on the economy since the progressive and devastating course of these diseases is a considerable societal burden, impacting social life and employment of patients as well as family members and carers. Therefore, the impact of these studies is mainly a long-term benefit. Moreover, any discovered mechanism can be explored in other neurodegenerative disorders such as Parkinson's disease, as each initially affects select brain neurons. Our hope is that treatment could be used to prevent the damage at early stages of the disease. Another possibility that we also aim to explore (as outlined in objective 3) is to reactivate dormant NSCs in the context of a diseased brain to explore whether particular brain functions could be ameliorated. Finally, we also hope to advance knowledge of basic molecular mechanisms underlying metabolic disorders such as obesity contributing to design more effective treatments.

How will you maximise the outputs of your work?

Findings will help first of all researchers in my team, and others at this REDACTED working on neurodegenerative diseases and fundamental basic mechanisms. Moreover, results will be made available to other scientists through publication in peer-reviewed journals, presentations at scientific conferences and meetings, and collaborations. This will allow us to share our novel findings not only nationally but also internationally, as we have previously done and is evident from my publication record over the years.

Under the previous/current project, we published 9 papers and 2 book-chapters of methods so far (and 5 more are currently being written), and we presented our findings at international scientific meetings. The mouse models developed so far and any valuable future models we will produce will be made available to other scientists interested in our models, as already done in the past and during the last 4 years.

REDACTED

Species and numbers of animals expected to be used

- Mice: 11000.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



The work has been organized in stages, first of all, a precise analysis of the genetically altered mice will be obtained from mice humanely killed, which will suffer only transient pain and distress due to either schedule 1 or non-schedule 1 killing. Once preliminary information is collected, we will then proceed with in vivo functional validation of the identified gene(s)/cell type involving brain injection and delivery of substances to modulate gene function, full recovery, administration of substances, monitoring of behavioural activity or metabolic phenotype, perfusion fixation under terminal anaesthesia.

For example, 60% of mice on a moderate protocol involving surgery, will experience surgery and injection of substances into CNS, full recovery, administration of substances, monitoring of behavioural activity or metabolic phenotype, perfusion fixation under terminal anaesthesia.

Or on another moderate protocol, approximately 15% of animals are likely to experience moderate levels of severity. This is because they will undergo surgery for intracranial administration of substances, full recovery, repeated blood sampling, dosing of substances, and behavioural tasks, perfusion fixation under terminal anaesthesia.

On a moderate protocol testing for metabolic dysfunctions where some animals could show alteration of fat/glucose metabolism, so about 80% of the animals are likely to experience moderate severity. They typically will undergo one of the steps outlined in the protocol, e.g. administration of substances, followed by one of the step options, then perfusion fixation under terminal anaesthesia.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Most of the animals will be used in a protocol with a mild severity limit. Animals produced under this protocol are not expected to exhibit any harmful phenotype. In the case of unexpected and unwanted harmful phenotypes, the animal will be humanely killed, or in the case of animals of particular scientific interest with an unanticipated harmful phenotype, advice will be sought from the NVS/NACWO.

Five protocols have a moderate severity limit; they involve ageing and/or surgery, or analysis of metabolic phenotypes. A smaller proportion of the animals will be used in these protocols, and we envisage that for many of the animals, the actual severity will be mild.

In particular, mice will be behaviourally tested at different stages, and so mice will be aged. These animals are not expected to show specific problems as they age. In some, after 12 months of age, learning will deteriorate quicker than normal mice. However, ageing animals will be monitored closely, including weighing, clinical examination and body condition scoring in consultation with the NVS, and any adverse effect due to a particular behavioural test will be dealt with accordingly.

In case of adverse effects of ageing that include skin and dental abnormalities, eye conditions, reduced organ function, tumours, as explained in each relevant sections of this PPL they will all be appropriately mitigated.

Other mice, instead, will be anaesthetised and have surgery to inject substances into their brains: some substances will mark nerve cells so that we can monitor and manipulate them; other substances will damage cells so that we can model disease processes. Animals that have had surgery are expected to recover quickly and will be given painkillers



and post-operative care so that any adverse effect due to surgery will be treated according to the effect presented.

Some mice on another specific protocol will be analysed for metabolic dysfunctions. Also, in this case, there are no significant adverse effects expected.

We have set up a Protocol to obtain tissue for ex vivo analysis. The majority of animals are likely to experience mild severity because they will be aged to the appropriate stage, and only a small percentage of mice will be aged beyond 18-20 months (and up to 28M). Care will be taken for this phase. Animals exhibiting any unexpected unwanted harmful phenotypes or in case the severity is likely to exceed moderate will be killed, or in the case of animals of particular scientific interest with an unexpected harmful phenotype, advice will be sought from the NVS/NACWO.

Finally, one last Protocol, combines ageing/behaviour and surgeries with the administration of substances; therefore, mice on this protocol will be monitored closely including weighing, clinical examination and body condition scoring in consultation with the NVS, and any adverse effect due to a particular behavioural test will be dealt with accordingly. Seemingly, for those undergoing surgeries, any harmful effect due to surgery will be treated according to the effect presented.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Most of the animals will experience no more than a mild severity, this includes:

breeding of genetically altered animals, which is expected to result in a phenotype with mild effects;

administration of anaesthesia except for the sole purpose of killing;

pharmacokinetic study where a single dose is administered and a limited number of blood samples are taken (totalling < 10 % of circulating volume) and the substance is not expected to cause any detectable adverse effect;

superficial procedures, e.g. ear and tail biopsies;

administration of substances by subcutaneous, intramuscular, intraperitoneal routes, gavage and intravenously via superficial blood vessels, where the substance has no more than mild impact on the animal, and the volumes are within appropriate limits for the size and species of the animal;

feeding of modified diets, that do not meet all of the animals' nutritional needs and are expected to cause mild clinical abnormality within the time-scale of the study.

A small proportion of animals will experience a moderate severity, this includes:

Surgical induction of CNS disease-relevant phenotype, full recovery, surgery for removal of devices (e.g. cannula for the implantation of osmotic minipump);



Ageing beyond 18-20 months.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 type of investigation outlined in this project cannot be carried out in cell culture as this lack information about the networks of neurons that are found in an intact brain. Furthermore, cell cultures or organoids do not replicate disease progression.

While other organisms such as yeasts, worms, flies and zebrafish are excellent models for studying the cell cycle and many developmental processes, mice are far better tools for asking questions about the immune, endocrine, nervous, cardiovascular, skeletal and other complex physiological systems that mammals share.

Also, the use of computer models, modelling, for instance, behavioural systems or processes, biochemical, or physiological events are not appropriate for the programme of work outlined in this application because many cellular parameters are unknown especially those regarding disease progression.

Manipulation of mouse genetics provides a unique opportunity to study the molecular basis of complex cell-cell interactions in vivo. The increasing availability of cell-type specific promoters gives extraordinary power to the generation and analysis of precise mouse models that undoubtedly will help to dissect the complexity of the brain. Lately, by combining precise mouse genetics and ex-vivo tissue analysis such as transcriptomic analysis (by RNA sequencing) together with bioinformatics we have been able to uncover unexpected findings regarding early changes occurring in neurodegeneration in select neuronal types. This will also allow, in the future, to compare these findings with diseased human tissues.

What was your strategy for searching for non-animal alternatives?

Cell cultures or organoids, computer modelling, other organisms such as yeasts, worms and flies.

Why were they not suitable?

The alternatives considered above are not suitable for the programme of work outlined in this application because they either do not replicate disease progression or because we do not know all cellular parameters, e.g. regarding disease progression; or because other organisms do not share complex physiological systems with mammals.

Once this knowledge is achieved, it will also facilitate the use of alternatives to a living protected animal in a regulated procedure.

Reduction



Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimate is based on our relevant experience working with small rodents such as mice. As well as power calculations.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Generation of very precise and specific genetically altered mice results in more defined, less variable and hence more relevant data. This correspondingly reduces the number of animals necessary to obtain significant results as well as improves the quality of life of the animal.

We will plan, conduct and record our experiments with the aim to publish our results according to the ARRIVE guidelines. In general, to reduce potential sources of variability and biases (e.g. sex, weight, genetic status, etc.) we will use, for example, blinding, randomisation in our experimental design where appropriate.

Where suitable mouse lines already exist, animals will be obtained from the relevant supplier. Otherwise, we will generate the required lines (including conditional knockouts) with the help of specialized services. This part of the programme will be implemented by the Transgenics Officer, who has the expertise required to deliver satisfactory results (i.e. healthy animals of the desired genotype). We now use CRISPR/Cas9a which should bring benefit both to the reduction of the numbers of animals used and the ability to create more refined genetic mouse models. Validation and characterization of edits introduced by CRISPR/Cas9a will be done on both the molecular and phenotypic level to assess their biological relevance. A variety of assays can be used, they will help to yield various types of information. These include characterization of the nature of the edit on the sequence level, biochemical level as well as on the functional level.

Efficient colony management ensures that only colonies that are actively being used are mated and produce animals. Those that are no longer required are cryopreserved and closed at the earliest opportunity. We now archive sperm as the main method of cryopreservation which reduces the number of animals required to secure a line.

Generally, we use power analyses to aid our experimental design and our own experience (25 years) as well as others from the literature. We will continue to use good practice in experimental planning, including statistics such as power and breeding calculations using current breeding figures to predict the number of matings required for experimental cohorts. We will measure production and breeding performance and ensure the minimum numbers of animals are used in the programme, as done so far. The statistical analyses (and software/code for implementation) that we use for our experiments are now well established and have been frequently peer-reviewed. Nevertheless, we continually strive to improve the analyses, and, take advantage of Statistical Consulting services, as well as online tools (such as the NC3Rs' Experimental Design Assistant).



Also, well-trained people will perform surgical methods. This, together with maximal care, will reduce the loss of animals recovering after an experiment.

If pilot studies are necessary to test a method or hypothesis, dose range finding, and/or to implement the 3Rs, they will be carried out using small groups of animals; based on our own experience as few as 2-5 animals per pilot study are sufficient to reach a robust decision point.

Where necessary, and following best scientific practice in the field, control groups (including, in some cases, sham surgical controls) will be used to help us interpret the effects of experimental interventions. For example, for some experiments necessitating the intracranial injection of a toxin or other agent designed to induce a subtle, disease-relevant phenotype, an important control would entail intracranial injection of vehicle. The number of animals undergoing surgical procedures in control groups will be kept to a minimum through (1) the use of 'within animal controls' e.g. injection of toxin/agent in one brain hemisphere, and injection of vehicle in the other hemisphere; (2) timely comparison of small groups of sham surgical controls with non-surgical controls to reach a robust decision point, e.g. if a difference were not detected, then it would not be necessary to use further sham surgical controls in that series of experiments; and (3) taking account of the published evidence.

Higher number of animals in the breeding protocol is because most of the mice are used to harvest tissues after schedule 1 method, or perfusion, exsanguination under terminal anaesthesia; or some mice having short breeding life because e.g. they develop an age-dependent obesity due to particular mutations; or for some experiments certain substances can only be administered to one sex (e.g. mifepristone cannot be administered to females because it blocks also the progesterone receptor).

What other measures apart from good experimental design will you use to minimise numbers?

Measures currently implemented

Generally, we ensure that the breeding of GA REDACTED be as effective as possible without compromising the genetic integrity of the lines. We normally use for breeding GA animals with no harmful phenotype. We usually obtain the maximum possible data from a single animal. For instance, perfusion-fixed tissue obtained for ex vivo histological analyses after addressing our primary aims on the particular protocol is archived for future studies. We will continue this in this project.

In addition, with the new established protocol of purification and sorting from young adult and aged mouse brain we have been able to perform analysis on <200 purified neurons, as well as on single neurons. This has allowed us to use very few mice per age/condition analysed and to reduce the overall number of mice considerably. To give an example, the ex-vivo protocol established allows to consider each animal as a biological replica, whereas standard protocols would require to combine 4-5 animals per each biological replica. We have now published these methods so that also the scientific community can benefit from them. We will continue to use these methods and refine them further.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the



procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

1. Animal models

a) We are creating genetically altered mice that allow us to delete/activate genes of interest in specific cell types and/or at specific stages in the life of the mouse. The mouse models described above in the project plan are the most refined for the intended purpose because by targeting only certain cells you avoid unwanted side-effects.

b). In general, the severity level is mild but there are some occasions when it is necessary to allow GA animals to age in order to sufficiently model the human diseases; for instance, this is the case with aggregates in AD. By leaving the mouse to age (typically up to 18-28 months) the phenotype reaches a point where we can assess the full extent of neuronal degeneration. However, these are very few mice, that might experience adverse effects of ageing.

2. Behavioural testing

The behavioural tasks we use include elevated plus maze, fear conditioning, radial maze, water maze that are in general not repeated on the same cohort of animals. We also use the novel object recognition, the open field (OF), CatWalk, and rotarod that are usually repeated on the same cohort of animals up to five times. All of these tasks are well established in the literature. Number of trials per day is based on published literature and our own studies. In general, the procedures involved do not give rise to adverse effects. Here are a few examples of the tests we perform:

Fear-conditioning test involves a mild foot-shock; sensory perception of the shock used in fear- conditioning has been determined in the past through shock threshold assessment, and the commonly used one gives rise to an annoying stimulus in the mouse that is still enough to generate an associative learning process. Classical conditioning in the brain has mostly been studied in two paradigms: Foot- shock fear conditioning and eye-blink conditioning. These two forms of conditioning are subserved by very different neural mechanisms. We will use the foot-shock paradigm, which covers the neural circuits of interest. At the moment there are no other paradigms that would effectively substitute for the foot- shock fear-conditioning paradigm. However, the foot-shock used in this paradigm is very mild and according to published literature (maximum of two or five conditioning trials co-terminated with 2sec 0.5 mA footshock, which does not lead to excessive vocalization (see Musumeci et al., J Neurosci 2009 • 29(32): 10131–10143). This is typically on one occasion during the life of the mouse.

The Radial maze testing involves that mice are food deprived approximately to 90% of their free- feeding body weight for the duration of the experiment (around 20 days). In general, this procedure is not giving adverse effects. However, in case of animals drop below 85% of their free-feeding body weight they will be switched to a regular diet, and humanely killed upon no recovery within 24-48 h. Although mice on this task can either be food or fluid restricted, we have been using the former throughout all our experiments, and we will continue to do so to be able to compare results.



Nonetheless, food and fluid deprivation or restriction are both considered to be acceptable procedures as long as suitable monitoring protocols are implemented, such as routine weighing and target weights. days). In general, this procedure is not giving adverse effects.

The Morris Water Maze (MWM) Involves that mice are placed in water of a comfortable temperature (22-25°C) and are trained to locate a platform just below the surface of the water (which is made opaque), and are left swimming for 60-90 seconds until they have found it. If they do not find the platform in 90 seconds, they are placed on it, then removed from the tank and dried. A potential adverse effect is hypothermia (drop in body temperature, reduced swimming speed) in this event mice will be removed from the WM and dried with towels. A heating pad will be available for mice exhibiting signs of stress. Moreover, if mice show fatigue /exhaustion (struggling to swim and to remain on the water surface) the trial will be immediately terminated. Rest period between trails will be increased and / or maximum trial time will be reduced.

The rotarod is a test to evaluate motor coordination, balance, and motor learning. In this test, mice are examined for their ability to maintain their balance on a rotating bar that accelerates 7.2 rpm/min. Over the course of each 5-min trial, the speed of the rotarod accelerates from 4 to 40 rpm. Suitable padding on the floor is provided to avoid injuries. The amount of time before a mouse falls from the rod is measured. Mice undergo three rotarod trials per day for two consecutive days with an intertrial interval of 15 min. There is very little chance of injury when a mouse falls off the rod because they would land on a mat, however animals will be checked for any injury before being returned to their cage.

The open Field is a test that offers a unique opportunity to systematically evaluate novel environment exploration, general locomotor activity, as well as an initial examination for anxiety-related behaviour in rodents.

Behaviour of GA mice is the ultimate readout in order to understand biological mechanisms. Therefore, we will continue to use different behavioural tasks and we will continue to refine them so to obtain meaningful results from the minimum required number of animals.

3. Appropriate surgical methods (which include short time surgery, aseptic environment) and post-operative care (such as analgesia, easy access to food and water) will help to minimize the occurrence of adverse effects. Animals will be given time to recover before any other step will be applied (postoperative recovery of animals and survival up to 16-weeks (typically 4-8 weeks)).

Other methods include:

Glucose tolerance test (GTT) and/or insulin tolerance test (ITT) to be carried out on adult mice. Prior to tests, mice will be fasted overnight or for 6hrs (GTT) or just 6hrs (ITT). For GTT studies, mice will be injected intraperitoneally (IP) with a D-glucose solution (2 g/kg body weight). For ITT, mice will receive an IP injection of human insulin at a dose of 1 U/kg of body weight. Venepuncture or venesection blood glucose concentration will be measured at a few different times after challenge using the commercially available blood glucose meter and test strips. Five to ten microliters will be withdrawn for each time point. However, no more than 10% of circulating blood volume will be removed at once or 15% in 28 days.



Different types of diets. Some of the mutants showing the metabolic phenotype will be fed different types of diets, such as high-fat diet to study the nature of obesity such as abnormally increased appetite, and/or consumption of food. However, this is only for short period of time before they are returned to their normal diet or killed experimentally.

Administration of substances. The substances to be administered should not in themselves cause pain, suffering, distress or lasting harm. Drugs/vehicles will be administered at established concentration/volumes and via routes most appropriate to mice as shown by other investigators or by our own experience. If testing a new drug where little or incomplete knowledge is available we will set conditions in a pilot experiment starting from the lowest doses.

Examples include the following:

Some mice will be given a substance that labels the DNA or the cell by routes suitable for the species and procedure (e.g. bromodeoxyuridine (BrdU)). In general, it will be by intraperitoneal injection (IP) or in the drinking water (1mg/ml) up to 7 days followed by a few-weeks survival before brain dissection after terminal anaesthesia.

To achieve induction or suppression of transgene expression we will treat some mice with drugs such as tamoxifen that per se has no toxic effect on mouse at well-established concentration. The administration of tamoxifen can sometimes lead to adverse effects, such as transient loss of body weight (<15%) and/or changes to body condition (e.g. piloerection) and behaviour (e.g. mobility, gait, posture). However, in our experience, systemic administration of tamoxifen using dosing regimens that induce robust transgene expression in the brain (e.g. daily intraperitoneal injections of tamoxifen at 30- 50 mg/kg (in corn oil), for 5 consecutive days) does not significantly impact on the animals' body weights, conditions or behaviours.

Some mice will be treated either with agonist-antagonist substances to activate or deactivate neuronal receptors such as neurotrophin receptors, related receptors, or related to this programme of work; or functional analogs (such as dexamethazone or corticosterone) to be able to study feedback inhibition in particular brain structures such as the hypothalamus of GA mice and respective controls; or other substances that mark cells (e.g. fluorescent dyes, biotinylated molecules); or substances that have potential for slowing, halting, reversing or otherwise ameliorating brain dysfunction in animal models of disease.

Typically, these compounds will be administered on one occasion only, or for 24 hours in the drinking water and no significant welfare, problems are expected in such a short time.

Some aged animals intracranially injected (after 4 or 8 weeks) or aged animals carrying the appropriate genetically modified alleles will be administered substances such as small molecules once per day for a prolonged period (for 3 to 4 months or up to 5 months if necessary) or twice daily for not more than a week, via the most appropriate route including oral gavage. All substances will be administered at doses known to be non-toxic, based on experience and dosages reported in the literature, and at volumes by following guidelines. All animals receiving substances will undergo routinely increased monitoring while under dosing regimen. Bodyweight and behaviour (e.g. lack of grooming, or coat condition) of all animals receiving substances will be regularly monitored. Oral gavage is a commonly used method of administration; it is performed with care by competent persons. Among oral administration methods, oral gavage is the most accurate and reliable method for administering substances into the gastrointestinal tract. This is because it eliminates



risks of variability in intake between individual animals. However, it is more invasive compared to other oral administration methods. Therefore, to minimise adverse effects, especially during chronic administration, we will use the smallest possible volume of a substance by not exceeding the maximum amount of 10ml/kg (10µl/g). We will investigate the properties of the substance, such as possible toxicity, from previous studies. We will apply the fewest possible number of doses so to minimise the risk of complications upon repeated gavages in the same animal. Animals will not be gavaged more than twice a day for more than a week. For chronic administration, it will be once per day for more than a month (for 3 to 4 months or up to 5 months if necessary). Moreover, adverse effects will also be minimised by the performance of the procedure by competent investigators or trainees.

In a minority of experiments, we may administer substances using a single osmotic minipump (or a similar device) to allow for slow/steady dosing with a profile that is not readily achieved through repeated and timed acute administrations. These methods are highly refined for the purpose.

Some mice will be subjected to micro-injection (into specific regions of the brain including hypothalamus, hippocampus, different cortical regions, amygdala and ventricles) of agents such as nucleic acid vectors, (including viral vectors packaged within viral particles), with, for example the Cre recombinase gene sequence inserted, or other recombinases, or shRNA (to silence genes of interest related to this programme of work); biocytin, visual beads, and neural tracers. These vectors/substances have already been tested by others and not reported to induce particular welfare problems.

Some of the virally transduced animals engineered to express a diphtheria toxin (DT) receptor on the surface of a specific cell type (after 1 or 2 weeks) will receive diphtheria toxin (DT). These animals are normal until exposed to DT, which acts as a potent inhibitor of protein synthesis and kills only those cells that express the DT receptor. This is highly refined methods to ablate only the desired cells in the brain.

As for the administration of substances such as mifepristone (a glucocorticoid receptor antagonist, that has been shown to produces clinical and metabolic benefits in patients with Cushing's syndrome), there are no adverse effects reported in literature at the dose used in this protocol and for several weeks' administration in the drinking water (literature and our own work) except that in mice with glucocorticoid induced metabolic changes leading to obesity will ameliorate the symptoms and help to validate mechanisms.

Some of the virally transduced animals engineered to express a G-protein coupled receptors, Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) on the surface of a specific cell type (after 1 or 2 weeks after intracranial transgene induction) will receive a drug-like small molecules such as clozapine-N-oxide (CNO) to modulate neuronal activity. These animals are normal until exposed to CNO, that for instance has been specifically shown to modulate feeding circuits (leading for example to increased food intake, meal numbers etc)., or other neuronal circuits. Drug-like CNO are relatively metabolically inert in rodents, and no side effects have reported when used at appropriate concentration in control animals lacking the engineered DREADDs. Likely route: intraperitoneal (IP) injections in the first instance, subcutaneous injections (s.c.) or in the drinking water if necessary.

The maximum frequency of injections (IP) will be up to twice daily and spaced approximately 8h from each other typically for a period of 2 weeks and no more than 3 weeks. If an inert substance such as CNO (at proven efficacy from the literature) is to be



injected more often, the period between injections won't be less than 5h and the duration of the experiment will not exceed a week.

In all cases of administration of substances or withdrawal of body fluids, we will use whatever technique is considered to be the most refined in terms of the scientific objectives and animal welfare.

Induction of CNS cell dysfunction

The induction of CNS cell dysfunction, by chemical means will likely change the animals' behaviour. We will regularly assess the behaviours, body conditions and body weights of animals after induction procedures. The behavioural changes will vary according to the CNS cell type(s) affected, the agent used to cause cell dysfunction, degeneration, and the time elapsed since the causative procedure. In the case of causative procedures targeted to the basal ganglia, the resultant behavioural changes will typically manifest as alterations to motor function (e.g. slower locomotion, subtle changes to gait), potentially relating to partial sensory neglect and/or cognitive deficits.

Behavioural changes can manifest within a few days of the causative procedure (e.g. after intracranial injection of neurotoxins such as 6-hydroxydopamine). Some behavioural changes may be subtle, such that they can only be detected after rigorous quantitative testing of the animals' behavioural performance.

In the case of animals receiving unilaterally-targeted procedures, changes in motor function may only manifest on one side of the body (e.g. a tendency to move in a certain direction, an asymmetry in forelimb use when rearing, and/or an asymmetry in the quality/quantity of grooming due to the partial loss of dopaminergic neurons affected by the lesion). From the literature, it is uncommon for these behavioural changes are reported to not significantly affect the animals' ability to eat, drink and groom. In most cases, the induced dysfunction, degeneration of the CNS cell population(s) will not be complete. In most cases, CNS cell dysfunction, degeneration will be induced by unilateral targeting, rather than by bilateral or systemic targeting. In the case of bilateral or systemic targeting for induction by chemical means, doses of the causative agent will be carefully titrated to ensure only partial coverage and that, accordingly, behavioural changes do not significantly affect the animals' ability to eat, drink and groom. Moreover, these mice will be used in one or two weeks after the procedure to achieve our objective.

Humane end-point

Routine health monitoring post-operation or during treatments using scoring sheets will help identifying early point of suffering such that humane end-point can be applied. Separate recording score sheets will be used for surgical (like stereotaxic injections) and non-surgical (systemic drug administration) procedures.

Individual housing

Typically, single-housing is not required unless there is no practical alternative (e.g. if only a single male or female of the desired genetic status is born in a given litter, or it is part of procedures like behavioural tasks (such as the radial maze); or occasionally when mice fight, or when stud males are temporarily removed from matings, or if group housing could compromise the clinical monitoring and/or treatment of an animal (as advised by the NVS). The duration of individual housing will be kept to an appropriate minimum. Where appropriate, individually-housed animals will be given extra enrichment (such as tunnels)



in their home cages. Where appropriate, animals will be re-housed with replacement cage mate(s) to provide companionship.

Cage enrichment

Some animals that have to undergo cognitive behaviour, due to concerns that complex environmental conditions will increase variation in the experimental results, will be provided only with environmental enrichment like nesting material.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

In the past century, the mouse has developed into the most useful mammalian model system for genetic research. This is not only because of its genetic and physiological similarities to humans, but also because of the ease with which its genome can be manipulated and analyzed.

While other organisms such as yeasts, worms and flies are excellent models for studying the cell cycle and many developmental processes, mice are far better tools for asking questions about the immune, endocrine, nervous, cardiovascular, skeletal and other complex physiological systems that mammals share.

Mice naturally develop diseases that affect these systems, in addition, certain diseases that afflict humans but not normally mice, such as cystic fibrosis, Alzheimer's disease, Huntington's disease can be induced by manipulating the mouse genome and the environment, see for instance (Saito T et al., Nat Neurosci 17, 661–663 (2014)).

For complex intercellular interactions in a developmental context such as the nervous system, it is essential to look at in vivo biology. This is the most efficient and most effective way of assessing the function of proteins in this context, and to manipulate their genes using molecular genetics technology.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We always strive to implement and refine procedures. In general, potential refinements include increased monitoring, post-operative care, management of pain and training of animals.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

NC3Rs online guidelines, practical information, and other online resources.

Asepsis guidelines (LASA ideally, Home Office at a minimum).

Laboratory Animal Science Association (LASA) that provides advices for care and welfare, education and training, ethics and policy and regulation of animal research.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Through literature updates, seminars, meetings, as well as interaction with NIO and NC3Rs Regional Manager, as done so far.



Explain the choice of species and the related life stages

The type of investigation outlined in this project cannot be carried in cell cultures as they lack information about the networks of neurons that are found in an intact brain. Furthermore, cell cultures or organoids do not replicate disease progression.

Manipulation of mouse genetics provides a unique opportunity to study the molecular basis of complex cell-cell interactions in vivo. The increasing availability of cell-type specific promoters gives extraordinary power to the generation and analysis of precise mouse models that undoubtedly will help to dissect the complexity of the brain. Using different life stages is required to carry out longitudinal analyses of brain function and behaviour at several pertinent life stages of the animals.



113. Neural regulation of central and peripheral circadian rhythms

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

The aim of this project is to investigate the neural mechanisms, from behavioural input to cellular physiology, that drive the mammalian circadian clock.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 circadian clock is responsible for setting and regulating both behavioural activity, sleep-wake cycles, through to internal hormonal rhythms. Disruptions in the functioning of the central circadian pacemaker has devastating effects on health e.g. depression, cancer onset and physical wellbeing. Thus, by understanding the way in which the circadian clock functions, we will be able to all begin developing novel therapies, both behavioural and pharmaceutical in improving over all health.

What outputs do you think you will see at the end of this project?



The key outputs will be adding to the knowledge of circadian clock function through journal publication, public engagement and external scientific meetings.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the first instance, the wider scientific community which will use outputs to inform the development of future projects. In the long-term, it can be envisaged that our outputs may also inform transnational research into circadian related disorders and their treatments in humans.

How will you maximise the outputs of your work?

We will aim to publish all data that is generated by this work, positive or negative. In addition, we will form collaborations, when natural, to support and increase the probability of generating outputs for publication and public engagement.

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. Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will be singly housed, and motion activity recorded using infrared sensors, for approximately 8 months. Mice may be injected, either i.p. or via surgically implanted cannulas, with pharmacological agents to disrupt or enhance the robustness of circadian rhythmicity. In experiments designed under a repeated measures protocol, this may be up to 5 injections per animals.

For measuring pupil light reflexes, mice will be temporarily restrained for up to 1 minute whilst pupil measurements are taken. Mice may undergo this procedure multiple times.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

There are little or no adverse effects to animals that will undergo behavioural and pupil light reflex recordings. Animals that have received injections may feel discomfort at the administration site, however this should be acute and last only 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 species)?



The overall severity would be mild, however any surgical procedures would be in the moderate category. Throughout the course of the project licence we anticipate only a very small proportion of mice undergoing surgery thus being placed in the moderate category. The proportions are likely to be 90% mild and 10% moderate.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 in vitro alone are insufficient to show a candidate cellular abnormality is relevant to a given disease. This requires translation of molecular and cellular mechanisms into behavioural and electrophysiological measures that can only emerge with intact whole nervous systems and whole organisms. Also, the complex physiology in our proposed projects involves, many of the neural, hormonal and behavioural mechanisms interacting with one another, necessitating the use of animal models.

This project aims to elucidate the underlying mechanisms by which circadian entrainment is possible in mammals. Entrainment involves a complex neural network that cannot be modelled in vitro and requires an intact physiological system. In addition we require complex structures, such as the eyes, to decode light information for processing by the circadian clock. Previously we have considered using neural 'clock' cells grown in in vitro, however these cells have proven difficult to work with and do not lend themselves in addressing our project aims.

The administration of pharmacological compounds either systemically or centrally may result in mild transient discomfort however, at present; there is no alternative route by which such drugs can be given. Further, cannula implantation is the only non-terminal method available that allows central drug administration and in vivo behavioural/physiological effects to be monitored.

In order to study the light induced responses of retinal photopigments and understand how they function as a network to regulate behaviour, an intact physiological system is necessary. Further, drawing out age related changes is impossible using current in vitro techniques, as the aging process is very complex and impairments are usually a result of a number of physiological alterations through time. Consequently, there is no realistic alternative to the use of intact animals to address this objective. In theory, it would be possible to undertake these experiments in humans; however, no subjects have yet been identified with impaired melanopsin activity.

Ageing is a complex and multisystem process. We are proposing to investigate this in an intact physiological system, which is currently impossible to do in the in vitro modelling.

What was your strategy for searching for non-animal alternatives?



We have considered using an immortalised circadian clock cell line; these cells are good for molecular work but are not suitable for investigating whole systems level physiology and complex neural networks.

Why were they not suitable?

These immortalised cells are not suitable for investigating complex behavioural responses and neural networks.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 statistical power analysis to ensure that the minimum number of animals is used for these experiments. Statistical significance will be set at $p < 0.05$ and power to > 0.8 .

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

In order to minimise the use and surgical stress on animals we will employ a within subjects experimental design. This will reduce animal numbers, as each animal will serve as its own control. In addition, we will use homozygous breeding pairs where animals are not required to be littermate controls. All comparisons will be made using a repeated measures ANOVA with statistical power analysis based on a significance level of $p < 0.05$.

Variability will be regulated by using littermate controls where appropriate and using stable REDACTED such as C57BL/6J Jax model.

What other measures apart from good experimental design will you use to minimise numbers?

We will tightly regulate our breeding strategy's as to minimise the unnecessary generation of animal stock.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?



Mice show excellent circadian behavioural responses and have been used for a number of years within the circadian biology field of research. They also provide the best model for transgenic manipulations. Hence, we propose the use of mice as data collected from our studies can be readily compared with that already published and, in parts, serve as control data when comparing with transgenic models. In addition, we will collect genotyping tissue from ear punches as oppose to tail clipping, whenever possible in order to minimise animal suffering.

To date, the mouse presents with the greatest potential for genetic modification over any other vertebrate species.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The only alternative to using transgenic models for these experiments would be to employ pharmacological, surgical or environmental manipulations. These have been used before; however, in our proposed approach the associated neurophysiological pain is much reduced. Examples would be where drugs have been injected intraocularly rupturing the eye capsule to silence specific photoreceptors. This method is significantly severer than the generation of a transgenic mouse line. Furthermore, genetic manipulation is a much more reliable tool for generating reproducible and clearly defined phenotypes, extending the utility of our observations. We will only employ intraocular injection in cases where no transgenic is available to address our research objective. Following intraocular injection animals will be required to recover from anaesthesia for a very short time (1 hour) and then culled. Pupil light reflex recordings will be taken during this 1 hour of recovery. The reason why we cannot measure pupil size with animals under anaesthesia is due to the lack of a stable pupil size under the influence of such drugs.

The monitoring of circadian molecular mechanisms can be achieved in *Drosophila* and will provide good robust evidence for their role in mammals; however do not provide detailed inferences on their physiological regulation by other complex efferent neural inputs such as feeding, social interactions. In addition, less sentient protected animals such as zebra fish, can provide detailed behavioural circadian outputs however are limited for understanding complex neural networks that are found in the mammalian brain.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animal suffering will be minimised throughout each experiment. During behavioural experimentation we will provide good husbandry and environmental enrichment, when this does not interfere with experimentation. Prior to experimentation animals will be regularly handled in order to acclimatise to the experimenter and restraint. All surgical procedures will be undertaken using aseptic techniques and appropriate analgesic administration will occur during and or post-surgery as required. Post-operative care will consist of pain management and regular monitoring (every 20 minutes for the first 2 hours and then at regular intervals as appropriate).

All experimenters will have superior training prior to conducting any experimentation. In the first instance, they will observe each procedure and then undertake these on their own whilst being supervised by the trainer. Only when the trainer is completely satisfied the procedure has been carried out competently will the experimenter be allowed to continue without supervision.



What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will consult the nc3r's website for up-to-date information as well as regular documents that are circulated through our AWEB.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The nc3r's website provides an outstanding resource of information. We will review this website on a regular basis for relevant information. In addition, our local AWEB provides us with an excellent platform, to discuss the 3R's, where this is a long standing item on every agenda.

Explain the choice of species and the related life stages

Typically, we will use adult mice. Mice are an ideal species due to the availability of transgenic models that present as excellent tools for research into physiology and disease. We use adult mice as these will present with the most robust and measurable circadian rhythms.



114. Neurovascular breakdown in ageing and disease and the development of novel vascular based therapies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Mice	aged, adult, juvenile
Rats	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

Neurovascular coupling is a mechanism that regulates blood flow in the brain. It is thought a breakdown in neurovascular coupling may be an important factor in disease and ageing, particularly dementia. This project license will investigate how neurovascular coupling is operating in healthy brain and how it goes wrong in disease. We will also develop new treatments to change baseline blood flow and/or neuronal activity to assess whether this slows down or stops progression of the disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Even though we have known about the existence of neurovascular coupling for over 130 years we still do not know how it works. As the breakdown of neurovascular coupling is becoming increasingly linked to brain disease, especially dementia it is more important



now than ever to understand firstly how it works in the normal brain as well as trying to understand how it is going wrong in disease. Due to the advent of new technologies it is now possible to target the vascular system for potential new therapies and this will form the third part of this project license.

What outputs do you think you will see at the end of this project?

Outputs from this project will primarily be in the form of peer reviewed scientific publications in leading neuroscience journals. These outputs will be across three general areas: basic mechanisms of neurovascular coupling, how neurovascular coupling goes wrong in disease and finally effects of neurovascular treatments to cure brain disease.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

There will be immediate, mid and long term benefits of these outputs.

Our research will help improve the use of brain scanning techniques that are used on human patients. By understanding exactly what these techniques are measuring in the brain we will be able to increase their accuracy and effectiveness. In the mid-term our research will also address the critical question of whether neurovascular coupling is breaking down in disease. If this is true, for example in Alzheimer's disease we may develop a new way of detecting the disease earlier. Early detection will allow more time for existing drugs to work and our results may isolate parts of the brain that need to be treated to slow down or even cure the disease. In the longer term if we can develop therapies that can slow down disease in our animal models of disease there would then be potential to translate these into human treatments.

How will you maximise the outputs of your work?

The project license holder and deputy already have a strong international collaborative network involving teams from New Zealand, Canada and the USA. Throughout the course of this project we will see to reinforce these collaborations by applying for international network grants to extend the scope of the research. We routinely publish all of our experimental results including negative findings. Indeed one of our recent papers showed that neurovascular coupling deficits reported in a mouse with Alzheimer's Disease may be an artefact. This finding although showing a negative result was really important to publish and share with the scientific community.

Species and numbers of animals expected to be used

- Mice: 2700
- Rats: 150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



We will use wild type control animals and Genetically Altered animals for this project. The GA animals either will have a disease (e.g. Alzheimer's) or be bred with proteins called opsins in specific brain cells that we can then stimulate using light with optogenetics. For the animals with disease, we want to assess whether neurovascular coupling is breaking down in disease and develop new treatments to test on them. For the optogenetics, we want to assess how a specific cell, for example an interneuron is involved in regulating blood flow in the brain and to potentially use stimulation of that cell in the treatment of disease.

Generally, acute non-recovery experiments will be used in the first instance to assess basic response and control stimulations. Then we will use chronic recovery experiments in separate animals that last in general for several months. These experiments may include a period of training or pre-treatment for the animal, followed by surgery to implant an imaging chamber to the skull. We have been doing these surgeries for a number of years and the animals recovery quickly. Then the animal will enter a phase of longitudinal recording from the brain, either with recovery anaesthesia or awake. Followed usually by a final acute non-recovery experiment before the animal is killed by schedule 1 method or transcranial perfusion to collect the brain for post mortem histology.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Some animals will undergo multiple surgical procedures and anaesthetics. In general the animals recover quickly and well from both procedures and anaesthetised imaging studies. Animals used in awake imaging protocols will need to be restrained, we will use positive reinforcement training in the first instance but food and water deprivation may be used. Some animals will have an altered diet (e.g. high fat western diet) for the duration of the study. Many of the chronic experiments under protocol two can last up to several months.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

In all acute anaesthetised experiments all animals the severity will be non-recovery. In our chronic experiments all animals that have cranial implants will be at a moderate severity. For the breeding protocols a vast majority of the animals will be in the mild severity band

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?



To understand neurovascular coupling in the brain, it is necessary to generate relevant biological data. The use of animals is therefore unavoidable. Rats and mice are the animal models of choice for research in this area due to the well-defined brain structure and function. That is, the sensory cortex these animals functions in a way very similar to human brains. Our expertise in using these animal models for research has been developed over a 20-year period. Consequently, the expertise and skills are already in place to ensure that the number of animals used is minimised by a consistently high quality of practice from the outset. We will also use specific mouse strains that reflect human disease such as Alzheimer's and they will provide important information about disease progression. As a general strategy, anaesthetised preparations will always be used in the first instance to address all objectives, and where new or modified techniques are employed. New techniques may then be introduced into the awake preparation. This will ensure that the number of animals required for chronic awake studies is reduced and that minimal stress is placed upon the awake animals.

What was your strategy for searching for non-animal alternatives?

As we need a functioning brain with a circulatory system there are no non-animal alternative for this work. In future artificial systems may exist for modelling of the responses we expect to see (called in silico medicine) but at present they are not available.

Why were they not suitable?

They cannot simulate the concerted action of millions of brain cells and a reactive vascular supply.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 are based on previous research and ongoing experimental work in the laboratory. In all experiments we routinely use statistical software and robust pilot data to calculate the numbers of animals need to reach appropriate statistical power in all our experiments. We can therefore achieve robust scientific results with appropriate groups sizes. All our grant applications have specific sections dedicated to the statistical calculation of group sizes and we have to adhere to ARRIVE guidelines with respect to randomisation and blinding in our experimental approach and analysis. Our estimate of animals is the minimal number of animals needed to generate scientific data that is statistically robust.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Our group have used the NC3R's Experimental Design assistant and G-Power software to generate group size estimates need to achieve robust significant results with appropriate groups sizes with the fewest animals needed to achieve appropriate statistical power. A



specific example of our experimental design is we have recently published where we used a 2-factor ANOVA repeated measure design to assess neurovascular breakdown in the J20 Alzheimer's mouse. As we were able to repeatedly measure the same mice over a period of 4 months (blood flow response to sensory stimulation) we were able to achieve robust results using only 10 animals.

What other measures apart from good experimental design will you use to minimise numbers?

We will liaise with our biological services unit to ensure all breeding protocols are efficient as possible and where possible all non-transgenic animals will be used as controls. All our group size estimates are based on robust pilot data. We are also actively collaborating with an academic team in New Zealand who use computer modelling of our data to understand potential mechanisms to explain our results. This modelling work will allow a more targeted approach in our future work. Finally, we are one of the leading laboratories in using multiple techniques in the same animal simultaneously and over multiple recording sessions. This allows the collection of higher quality data from fewer 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Our approach will be to use non-recovery terminally anaesthetised animals in the first instance to refine experimental approaches. Then we will use chronic experiments where we can repeatedly take measurements from the same animal over many months. This reduces the number of animals needed compared to using multiple animals at separate time points. We carefully train all staff before they are allowed to perform experiments and animals are constantly monitored during studies to ensure suffering and stress are kept to an absolute minimum.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We have to perform longitudinal studies for this project as we need to assess disease progression as part of our studies and develop potential new therapies as part of the project. We cannot use less mature animals because neurovascular coupling isn't established in very young animals and our main disease of interest is Alzheimer's which only occurs in adult or old animals. In our opinion the rodent model is the least sentient animal we can use while still being able to compare to results seen in humans

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?



We already operate high standards in monitoring and post-operative care of our animals. We have daily contact with the Biological Services Unit and the named animal care and welfare officer to pick up on the earliest signs of potential distress in our animals.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will keep up to date with the NC3R's ARRIVE guidelines and update members of the team in all aspects of best practice.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The project license holder is a member both the REDACTED Animal and Welfare Ethical Review Body to keep up to date with new developments and best practice. A member of our research groups has held a grant from the NC3R's.

Explain the choice of species and the related life stages

This project will use young, adult and aged rats and mice.

A key aspect of this project is to assess how neurovascular coupling is changing in health, disease and ageing. Therefore, we need to perform experiments across the animals lifespan. We chose the rodent model as we have 20 years experimental experience and it is the lowest form on sentient life that have a comparable brain and circulatory system to that of humans.





115. New therapies for mitochondrial optic neuropathies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The overall aim of this project is to develop and test new therapeutic interventions in mouse models of inherited mitochondrial optic neuropathy.

There are two key elements to the experimental strategy: firstly, to characterise the abnormalities in genetically modified mice that arise from alteration in genes that cause optic atrophy, and secondly, to determine the effectiveness of 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?

Inherited optic neuropathies are genetic conditions in which the optic nerve is damaged with the loss of cells called retinal ganglion cells leading to atrophy of the optic nerve. There are currently no effective treatments. Since the commonest form of inherited optic



neuropathy is dominant optic atrophy, with an incidence of 1:10,000 to 1:50,000, the problem is of significance to society. The importance of this work will be that it will generate data that will lead to new treatments for optic neuropathy being rolled out to patients. The project will evaluate new potential treatments specifically for the protection and repair of retinal ganglion cells. The project will provide supportive evidence for developing early clinical studies of novel therapeutic interventions and predict their likelihood of success in man. The beneficiaries of the project will ultimately be patients with inherited optic neuropathies. Novel treatments have the potential to have substantial impact on the quality of patient care in this disease and subsequently on the patients' quality of life. In achieving these benefits, we will also generate a deeper understanding of the mechanisms of development of the disease, which will also lead to potential benefits in diagnostic testing, early intervention and potentially even prevention.

These benefits are worthwhile because in the developed world two out of three blind people have retinal disease, with 20% of these due to retinal ganglion cell degeneration and optic atrophy.

What outputs do you think you will see at the end of this project?

The project will generate new information and publications and may see new therapies that can be developed further for the benefit of patients with mitochondrial optic neuropathies.

Knowledge generated in this project will also contribute towards future socio-economic impact as new data may be used to develop markers to improve diagnosis and track disease progression. Better understanding of the underlying mechanisms that drive retinal ganglion cell loss in these conditions will potentially lead to improvements both in the short term (public awareness, mechanistic discovery) and long term (therapeutic target development) for a significant number of individuals in the UK and worldwide.

This project will also generate peer reviewed publications in international journals, further grant funding applications, conference platform presentations and posters, and new collaborations. The novel therapeutic strategies are all likely to be suitable for intellectual property protection if they are promising and this will be sought.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The expected benefits of this research will be realised as the project advances, and will be pre-clinical data for novel therapeutic interventions in optic neuropathy. The project will provide supportive evidence for developing early clinical studies of novel therapeutic interventions and predict their likelihood of success in man. The beneficiaries of the project will ultimately therefore be patients with inherited optic neuropathies. Novel treatments have the potential to have substantial impact on the quality of patient care in this disease and subsequently on the patients' quality of life. In achieving these benefits, we will also generate a deeper understanding of the mechanisms of disease, which will also lead to potential benefits in diagnostic testing, early intervention and potentially even prevention. The realistic time frame for benefit for patients is 5 to 10 years.

How will you maximise the outputs of your work?



I am already establishing new national and international collaborations and will continue to do so throughout the project. I am actively seeking to utilise and share the mouse models I work on with colleagues who have promising therapies that require pre-clinical data. I am also seeking out industry partners to further these goals.

I will work with third sector charities and patient support groups to disseminate the results of our research and contribute to local and national events (including scientific conferences and patient groups). I am regularly invited to speak at patient events and this will be an ideal dissemination opportunity with the interested community of patients. Public awareness will also be enhanced through open access publication of results, and by dissemination of the results. I will also continue to work with both schools and adult audiences to increase the general public understanding and awareness of mitochondrial dysfunction and the devastating effect it can have on vision loss.

I will make timely publication of our results in high impact, open access journals a priority. I will deposit any large data sets in central open access repositories.

The research team will attend national and international conferences . These meetings will allow us to meet other academics, patients, health care workers, government policy makers and third sector investors and let them know the results of our work. I will engage in discussions to explore the potential for commercialisation and assess any outcomes that should be protected as intellectual property. In this way if there are discoveries made that could help patients in the future we can ensure that we maintain a lead in this field.

Species and numbers of animals expected to be used

Mice: 600 mutant mice per annum over 5 years, or 3000 mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, mice will be bred, maintained, some will be aged and phenotyped and some enrolled in therapeutic intervention studies. Those in the intervention administration study will have phenotyping before and / or after intervention.

The optimisation of the GA breeding strategy is key in reduction of the numbers used under this project. The project requires both wild type and heterozygous mutant mice- hence we have to breed wildtype and heterozygous mice as well as cross heterozygous mice with other heterozygous mice. All mice generated are valuable to the project.

Within each experimental cohort the minimum number of animals will be a minimum of 3 per genotype (wild type versus heterozygous versus homozygous mutants). However, in order to achieve statistically significant results these numbers may need to be increased by multiples, depending on the magnitude of the effect/ phenomenon being observed. Statistically meaningful data comparing wild type phenotype to heterozygous or homozygous mutants will be obtained. The ideal cohorts will comprise littermates, or will have been age and sex matched.



Phenotyping data will be collected which is qualitative and descriptive, including photographs of the eye. Where measurements are made for quantitative data care will be taken to use the minimum number of animals in order to obtain a statistically significant result and reproducibility.

The phenotyping will typically involve non-invasive assessment of ocular and retinal and optic nerve structure and function. Photographs and scans of the retina and optic nerve will be taken and vision assessed by psychophysical and electrophysiological tests. Administration of substances as therapeutic interventions will be either drops, subconjunctival injection, intraocular injection (as is often the case with medicines used to treat patients with retinal disease) or oral. Novel compounds would typically be tested at two or three dose levels around the expected pharmacological dose, using initially no more than 3 animals per group. For comparisons using larger groups, group size will be estimated by power analysis. Unless there are good reasons otherwise, designs will compare several compounds and/or dose levels and factorial experimental design will be used when applicable.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Mouse models of inherited optic neuropathy generally correlate well with human disease and therefore enable us to get better understanding of the disease mechanisms and potential routes to treatment. Although the genetic changes and diseases are chronic in duration, animals do not normally show any alteration in behaviour or feeding or significant widespread problems and most animals will experience very low severity, namely sight loss. Sight is not a main sense in mice as they depend much more on whiskers.

However, in order to reduce impacts and adverse effects for the animals the research plan has sought to refine and minimise animal suffering by the incorporation of the mutual-exclusivity of some of the procedural options, which prevent unreasonable levels of cumulative severity from occurring. The range of tests outlined are mostly non-invasive, and as such the severity of the testing is 'mild'. Where a test is more invasive it will incorporate anaesthesia, with or without recovery. Many of the tests, such as fluorescein angiography, electroretinogram recordings from the retina and optic nerve and imaging the retina are considered non-invasive in humans. In this work animals will need to be immobilised and pain relief will be administered. The tests will be carried out under the same session of general anaesthesia or sedation, thus minimising stress and potential harm to the animal. This also reduces handling, testing times and general anaesthesia exposure. In summary, all semi invasive or invasive procedures will be carried out aseptically. Peri- and post-operative analgesia will be provided; agents will be administered as agreed in advance with the NVS. Animals will be monitored until a full recovery is made from the anaesthetic. If clinical signs are exhibited post-anaesthesia, further monitoring will be undertaken using the Health Monitoring Score Sheet (appendix 1). In the uncommon event of post- anaesthesia 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

The administration of substances, or interventions, are of low severity in the main, although injection in the eye may be moderate and carried out under general anaesthetic and analgesia.



Where animals have to be housed separately they will receive environmental enrichment in their cages, with tubes and nesting in which they can hide as well as chew sticks as an activity item. In previous work, we also refined the experience of the singly housed animals by using raised lids in the cages so they could climb around and see the animals in their neighbouring cages. We will also ensure to take care not to alarm the mice with sudden noises or movements in the holding room.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

75% mild, 25% moderate.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 whole animal visual system and retina and optic nerve pathophysiology is needed to test the mechanisms of disease and to conduct pre-clinical trials of novel therapeutic interventions.

Investigating the complex processes involved in inherited optic neuropathy, which consists of many different cellular and tissue interactions in the retina and optic nerve, requires animals to establish the outcome of these processes in living animals. Non-animal alternatives cannot reveal the complex responses generated as a result of the administration of therapeutic interventions, and can only take us part of the way towards understanding the ramifications of any new treatment. Animals have to be used because it is not possible to remove tissue from the eyes of living patients who may be tested with new therapeutic agents without any data on their toxicity or effectiveness. The whole animal model provides the only way of assessing distant neurological effects of mutations in the causative genes.

What was your strategy for searching for non-animal alternatives?

My research utilises cell lines and primary cell culture extensively on an ongoing basis and I shall continue with this research in addition to the mouse work applied for.

Why were they not suitable?

Cell and culture models, ex vivo and in vitro, are suitable for some of the assays. The retinal tissue from mutant mouse is needed to provide more physiologically relevant data.

Reduction



Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The research requires the use of both wild type and heterozygous mice and hence no mice are wasted as a result of our breeding strategy. The number of animals used will be minimised by careful design of experiments using contemporaneous control groups and statistically appropriate group sizes, which includes between 4-8 mice in each group. To minimise animal usage we will investigate the cellular effects of therapeutic interventions in vitro by establishing cell lines before using these agents in the mice. The use of tissues for organ and cell culture from genetically modified and wild type control animals will allow complex molecular and biochemical experiments to be undertaken and will minimise the use of live animals.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We have based the numbers on previous experience with pre-clinical trial of a drug used in mitochondrial optic neuropathy. The online NC3Rs Experimental Design Assistant has been used to check our assumptions and predictions.

What other measures apart from good experimental design will you use to minimise numbers?

The optimisation of the breeding strategy is key in reduction of the numbers used under this project. The project requires both wild type and heterozygous mutant mice- hence we have to breed wildtype and heterozygous mice as well as cross heterozygous mice with other heterozygous mice. All mice generated are valuable to the project.

Within each experimental cohort the minimum number of animals will be 3 per genotype (wild type versus heterozygous versus homozygous mutants). However, in order to achieve statistically significant results these numbers may need to be increased by multiples, depending on the magnitude of the effect/ phenomenon being observed. Statistically meaningful data comparing wild type phenotype to heterozygous or homozygous mutants will be obtained. The ideal cohorts will comprise littermates, or will have been age and sex matched.

Phenotyping data will be collected which is qualitative and descriptive, including photographs of the eye. Where measurements are made for quantitative data care will be taken to use the minimum number of animals in order to obtain a statistically significant result and reproducibility.

Novel compounds would typically be tested at two or three dose levels around the expected pharmacological dose, using initially no more than 3 animals per group. For comparisons using larger groups, group size will be estimated by power analysis. Unless there are good reasons otherwise, designs will compare several compounds and/or dose levels and factorial experimental design will be used when applicable.



The sharing of tissue will be an important part of our strategy because we can carry out many experiments on cells from tissue.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice are suitable as the chosen species for this project because they have a well-characterised genome, considerable data is available about their normal visual function and physiology and it is possible to generate mice with specific genetic modifications to study. The time taken to generate the cohort of mice required in the experiments is relatively short. The mouse eye, especially the retina and optic nerve, has considerable homology to the human eye.

The methods comprise examining the mouse to measure its visual and neurological function. This is done in a way that is very similar to a human eye clinic assessment - such as measures of visual acuity, retinal and optic nerve appearance and imaging by special cameras through a dilated pupil and assessment of function through electrophysiology. The methods are mainly observational or non interventional.

The administration of potential novel therapeutic interventions will be either by eye drops, eye injection, or through food or water.

All animals will be closely monitored for distress and signs of ocular inflammation and appropriate steps to minimise harm to the animals will be taken following discussion with the Named Animal Care and Welfare Officer (NACWO) / Named veterinary Surgeon (NVS).

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mouse models are the least sentient compared to other species, whilst still correlating well with human disease and allowing more depth to probe disease mechanisms because of the availability of reagents and ability to genetically alter mice. Although the genetic changes and diseases are chronic in duration, animals do not normally show any alteration in behaviour or feeding. Sight is not a main sense in mice. These models have an excellent track record in preclinical testing of agents which are now used in man.

We study the effect of genetic mutations that lead to optic atrophy and blindness in humans and use mice which have the same genes mutated. This results in loss of vision but in mice the visual loss is not as significant since the mouse is less reliant on vision than humans.

However, to study the damage we need adult mice and the loss of vision often only advances with age. Other species would not mirror the human retina quite so well and the



genetics of some species makes them unsuitable. Pre-clinical testing of new therapies also requires them to be explored in a mammalian retina. The testing is delivered over a period of days, weeks or months in some cases, by eye drops, food or water or injection and therefore animals that have been terminally anaesthetised are not suitable.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The research plan has sought to refine and minimise animal suffering by the incorporation of the mutual-exclusivity of some of the procedural options, which prevent unreasonable levels of cumulative severity from occurring. The range of tests outlined are mostly non-invasive, and as such the severity of the testing is 'mild'. Where a test is more invasive it will incorporate anaesthesia, with or without recovery, for example, Protocol 3. In this protocol, all the tests, such as fluorescein angiography, electroretinogram recordings from the retina (ERG), and retinal and optic nerve (a battery of test considered non-invasive in humans; animals will need to be immobilized; pain relieve will be administered to prevent electrode placement causing any discomfort) will be carried out under the same session of general anesthesia (GA) or sedation, thus minimizing stress and potential harm to the animal. This also reduces handling, testing times and GA exposure.

Where animals have to be housed separately they will receive environmental enrichment in their cages, with tubes and nesting in which they can hide as well as chew sticks as an activity item. In my last PPL, we also refined the experience of the singly housed animals by using raised lids in the cages so they could climb around and see the animals in their neighbouring cages. We will also ensure that the care staff and relevant PIL holders do not alarm the mice with sudden noises or movements in the room.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Published guidelines on the NC3Rs website provide a regular source of up to date guidance.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I am part of a community of researchers with shared experience and knowledge of animal work and we share data and techniques and attend training both locally and nationally. There is a regular review of methods ongoing and the 3Rs are highlighted by seminars and workshops and conference events. The online 3Rs training provided by the NC3Rs is incorporated into practice regularly.

Explain the choice of species and the related life stages

I plan to use mice which are genetically altered to carry mutations which model human mitochondrial optic neuropathies. It not possible to achieve the objectives of this project without using animals for the following main reasons:

Firstly, the tightly controlled genetic background and environment of the mice is impossible to achieve in any human clinical study.



Secondly, investigating the complex processes involved in inherited optic neuropathy, which consists of many different cellular and tissue interactions in the retina and optic nerve, requires living animals to establish the outcome of these interactions in living animals. Non-animal alternatives cannot reveal the complex responses generated in response to the administration of therapeutic interventions, and can only take us part of the way towards understanding the ramifications of any new treatment. Thirdly, animals have to be used because it is not possible to remove tissue from the eyes of living patients. Fourthly, it is not ethically or otherwise possible to administer new therapeutic agents to human subjects without sound data from animal studies.

Lastly, the integrity of the visual system is particularly important if we are to provide accurate assessments of the degree of retinal ganglion cell recovery. The whole animal model provides the only way of assessing distant neurological effects of mutations in the causative genes.

I will use adult and juvenile and embryonic life stages. Adult mice are needed for breeding, and ageing. Juveniles can be aged and with age express more of the relevant phenotype. Embryonic life stages can provide cells for ES work and this can be very useful for disease modelling and therapeutic testing.





116. New Treatments for chronic ocular 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

No answer provided

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 is the aim of this project?

The aim of the project is to develop genetically-targeted therapies for chronic ocular conditions such as age-related macular degeneration.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Age-related macular degeneration (AMD) is the most common cause of blindness among the elderly in the industrialised world and accounts for 8.7% of all cases of blindness worldwide, particularly in people older than 60 years (Wong 2014). AMD is most prevalent in populations of European ancestry with approximately 1–3% of the total population suffering from an advanced form of AMD REDACTED



In the UK, the Royal National Institute of Blind People (RNIB) estimate there are around 600,000 people with sight loss due to AMD, with 70,000 new cases each year diagnosed, and this is expected to more than double to 1.3M by 2050.

The disease is caused by damage to the macula, which is the part of the eye needed for sharp, central vision. AMD is a complex, multifactorial disease which is difficult to manage and is classified into early, intermediate and late stages (Khandhadia 2012, Ferris 2013). AMD includes two morphological sub- types: neovascular AMD and the atrophic form, also called geographic atrophy (GA) (Chakravarthy 2010). Whilst there are highly effective biological therapies for the neovascular (or wet) form, there are no currently approved treatments for the atrophic (or dry) form.

REDACTED

What outputs do you think you will see at the end of this project?

The main outputs from this project will be the generation of data to support the progression of gene therapies programmes to clinical evaluation in chronic ocular disease. The data will either be supporting clinical development directly (e.g. will form part of the nonclinical data package for regulatory submission to conduct a clinical trial) or indirectly through the development of the gene therapy platform for future therapies.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

These outputs may be used by REDACTED to progress its gene therapy programmes by supporting clinical trial applications/approval in the short term (2-3 years per project) with the hope of successful treatment of dry AMD patients through clinical development and ultimately to market in the mid-long term (3 – 10+ years). It may also be used by REDACTED to support the development of its gene therapy platform, which may generate Intellectual Property in the short term and lead to improved therapies in the longer term. These outputs may also be presented at scientific congresses and published in peer-reviewed journals thereby benefiting the field as a whole throughout the term of this project and beyond.

How will you maximise the outputs of your work?

Data from these studies may be presented at scientific congresses or workshops (posters or talks) and published in peer-reviewed scientific journals and patent applications, thereby benefiting the entire field.

Species and numbers of animals expected to be used

- Mice: 1500
- Rats: 480

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, each mouse will receive a single subretinal injection of vehicle or vector under general anaesthetic. Blood samples (approx. 50uL for mice) may be taken at baseline and up to weekly during the 4 week procedure. Blood samples (approx. 100uL for rats) may be taken at baseline and up to weekly during the 4 week procedure. (see Table 1 in Step 1 for volumes) after which time the animal will be euthanised by an appropriate Schedule 1 procedure. In some cases, the animal may be anesthetized up to weekly for non-invasive ocular imaging (fundoscopy, OCT). In some cases, the animal may be terminally anesthetized for ocular imaging and/or terminal blood sampling, followed by Schedule 1 method. Tissues will be taken post-mortem for analysis.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Animals are expected to have pain due to the subretinal procedure that will be transient (days) and effectively treated by local and/or systemic analgesia. Artificial tears are applied during the subretinal procedure to counteract eye dryness. AEs are likely to be very uncommon (1-5%) with a maximum severity category defined as moderate. AEs associated with subretinal injection may include subconjunctival or vitreous haemorrhage (routinely self-resolved and do not require treatment), endophthalmitis (treatable with topical antibiotic), and on rare occasions the development of cataract or retinal detachment.

All animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Regarding the transient retinal detachment due to subretinal injection, mice are nocturnal animals with poor visual acuity. Therefore, it is accepted that they can cope with loss of vision without any appreciable detriment. Uncommonly animals that fail to do so or exhibit signs of pain, distress or of significant ill health, as assessed by the NVS, will be killed by a Schedule 1 method. Any animal not fully recovered from the surgical procedure within 24 hrs or showing general signs of ill health that are likely to exceed the limit of severity (moderate category; such as piloerection, lethargy, up to 15% body weight loss, grimace score reading of pain – see Table 2 above), 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 species)?

Most animals are expected to experience up to moderate severity due to the surgical procedure, however this is expected to be transient and will be alleviated by the use of anaesthesia during surgery and analgesia post-surgery.

What will happen to the animals at the end of the study?

- Killed

Replacement



State what non-animal alternatives are available in this field, which alternatives you have 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 currently use in vitro cell-based systems for much of our work to screen and characterise AAV vector candidates prior to in vivo testing and have looked into replacing the need for animal work through the use of more complex in vitro systems such as 3D organoid cultures and patient-derived induced pluripotent stem cell (iPSC) models but these are currently not suitable for this purpose. Assessing both AAV vectors and a complement-targeted therapeutic approach requires a fully functional ocular/immune system that cannot currently be modelled in vitro. However, we will aim to reduce the number of animals used in testing by substituting in vivo models to show efficacy of treatment with in vitro readouts, such as in vitro assays for complement activation. We will also minimise the number of rodents to test in vivo by implementing in vitro screening of treatments prior to animal studies.

What was your strategy for searching for non-animal alternatives?

We have considered complex in vitro systems (in which the retinal architecture is represented) such as 3D organoids ('retina in a dish') primary RPE and patient-derived induced pluripotent stem cell systems.

Why were they not suitable?

Assessing effectiveness of both AAV vectors and a complement-targeted therapeutic approaches requires a fully functional ocular/immune system which are not present in current in vitro/ex vivo models. However, we will continually monitor this field with the intention of replacing animal studies with non-animal alternatives if suitable.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers have been estimated based on previous studies with AAV vectors conducted by Gyroscope therapeutics at CRO's and academics, showing appropriate effect sizes and statistical significance. Power calculations are routinely employed to check that the number of animals per group will demonstrate statistical significance. . If internal data is not available, data from published literatures can be used as a source for power and sample size calculation. The number of studies per year is based on current and project work plans but may be lower if alternative methodologies to replace such studies are developed over the next 5 yrs.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?



The use of prior data as stated above will allow selection of appropriate group sizes likely to produce statistically-meaningful data, which will reduce animal numbers needed for this work. In vitro screening to select vector candidates will also be conducted prior to animal work using several techniques, including flow cytometry, western blotting and ELISA. Only the lead in vitro screened vector candidates will be tested in vivo to reduce the numbers of animals used..

As part of our Experimental Design, we will use the minimal number required per group that gives valid data based on our experience with similar mouse expression studies using this AAV gene therapy platform. Power and sample size calculation will be used to ensure that detection of significant difference is achieved with the smallest sample size.

What other measures apart from good experimental design will you use to minimise numbers?

In many cases, the numbers of animals required will be reduced by longitudinal measurement of responses, e.g. by serial blood samples or by optimised protocols for intravital imaging. Longitudinal usage provides essential information on the effect or not of any treatment, while at the same time reducing the number of animals required to obtain such information.

Unnecessary variation in the animal cohorts that could result in requiring more mice will be minimised by use of gender and age-matched controls housed under identical conditions.

Pilot studies will always be employed when the outcome of the study cannot be predicted with any certainty (such as first-time use of new platform/technology or therapeutic transgene or change in surgical procedure (changing from unilateral to bilateral subretinal administration, for example)). This will ensure dose and animal numbers can be optimised before embarking on any animal intensive studies, thus minimising the overall use of animals. Care will be taken to harvest animal tissue required for developing assays for assessment of in vivo studies from control animals from previous studies to negate the need to cull animals for this purpose and reducing animal use further.

As an example of optimisation of our animal approaches we may assess the impact of bilateral subretinal administration of AAV vectors. We currently administer AAV vectors by subretinal unilateral injection to mice. To reduce the number of mice used per experiment we may assess the impact of bilateral subretinal administration of AAV vectors on animal welfare (i.e. body weight, food and water consumption) and behaviour and compare this to baseline data generated by our current unilateral administration of AAV vectors.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The rodent is the ideal organism for these investigations for three main reasons:



Firstly, it is the long-standing choice for gene therapy research with lots of historical data available. This is due to the structural similarities between the target retinal/associated ocular structures in the mouse and human (i.e. similar sclera, choroid, RPE, photoreceptor (PR) layer, outer and inner nuclear layers, ganglion/nerve fibre layers, inner limiting membrane), and commonly used laboratory strains of mice are phenotypically and genetically characterized and do not show common retinal degeneration and mutations leading to retinal disease (Chang 2013). It is therefore recognised as the best first species to undertake ocular research in.

Secondly, there is a large number of well-characterised models and reagents available permitting the thorough, incisive, and comprehensive obtainment of information from the experiments undertaken. Thirdly, there are highly effective methods of longitudinal monitoring, e.g. an array of well-established methods used in human clinical eye examinations have been adapted for use with rodent eyes to assess the retina in a non-invasive manner. These methods include indirect ophthalmoscopy and optical coherence tomography (OCT), all of which will contribute to the reduction of the numbers of animals required. Such techniques are also focused on animal welfare, e.g. better imaging stations and methods requiring shorter times under anaesthesia.

Negative control groups (baseline groups) will be minimised whenever statistically feasible.

References

Chang et al., (2013) Survey of Common Eye Diseases in Laboratory Mouse Strains. *Invest Ophthalmol Vis Sci.* 54 4974–4981.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice are the preferred animal model for the evaluation of ocular gene therapies as the retinal architecture is very similar to Man despite the lack of a macula. Primates, who do have a macula, are not used instead for ethical reasons. Mice therefore represent the least sentient suitable species for these studies.

More immature stages of the mouse cannot be used for two reasons: firstly, the retina is still developing in the neonatal mouse, therefore the anatomical structure does not represent the therapeutic target (mature retina). Secondly, the vector must be administered by subretinal injection which in the small mouse eye causes a level of procedural damage. This is exacerbated in the neonatal eye.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Subretinal injection is a clinically established route of administration of therapeutic agents in humans. We will use the state-of-the-art technique and equipment adapted to rodents. We will consistently monitor the literature to ensure that we are using the most up to date and refined approach.

To minimise discomfort of repeated procedures such as anaesthesia, we will combine treatments under a single anaesthetic event wherever possible. The anaesthesia will



preferably entail the use of inhalation agents whenever possible. Least invasive route of substance administration and needle gauge will be used where possible.

All animals will be monitored, with additional monitoring post-vector administration. All animals will receive appropriate operative care in terms of anaesthesia and pain management both during and after the procedure.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We expect all recovery procedures will be carried out aseptically (see the Appendix 4 HO Minimum Standards for Aseptic Surgery, as well as other guidelines e.g. LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery). Peri-operative analgesia will be given and maintained after the procedure for as long as is necessary to alleviate pain. Animals will continually be monitored for signs of pain and distress, especially post-operative, by use of the grimace scale;

<https://www.nc3rs.org.uk/sites/default/files/documents/Guidelines/MGS%20Manual.pdf>.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Through working very closely with the REDACTED and by maintaining an awareness of the National Centre for the Replacement, Refinement and Reduction of animals in research (NC3Rs website - Arrive guidelines; Animal Research: Reporting of In Vivo Experiments), best practises will be continuously adopted.

Explain the choice of species and the related life stages

This data cannot be generated with current in vitro systems. Since AAV vectors have been shown to transduce the mouse retina effectively following subretinal injection, with a similar tissue distribution as primate, the mouse has been shown to be an effective system for the evaluation of gene therapy vectors.

Subretinal injection can be performed on younger animals (neonatal) however the subretinal procedure is challenging and is associated with a higher degree of procedural damage which could confound results. The adult mouse is therefore the best model.





117. New vaccines and anti-cancer immunotherapies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

The overall aim of this program of work is the discovery and development of new therapies and therapeutic combinations for the treatment of solid tumours and of new vaccines for infectious disease prevention.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 generate data describing the safety, efficacy and tolerability of potential new vaccine candidates against several infectious diseases. Similar data will be generated for potential new anti- cancer agents.



The data generated during this project will be used to select the strongest candidates for potential new vaccines that will prevent disease. Likewise, new anti-cancer agents will be identified with the potential of increasing the range of medicines available in the fight against cancer.

The field of anti-cancer microbial therapies is just beginning to develop, and the data generated in this project has the potential to influence the progress and direction of the entire field. Through scientific publications and patent applications such data will advance everyone's knowledge of how the immune system fights tumours and how this can be helped by bacteria.

What outputs do you think you will see at the end of this project?

This project will result in safety, efficacy and tolerability data for potential new vaccine candidates against several infectious diseases and for potential new anti-cancer agents.

The data generated during this project will be used to progress the strongest candidates into clinical development with the intention of developing marketable products. Data from this project will be also be used for filing new patents and will be disseminated through the patent publication pathways.

In addition to patent applications, scientific publications and conference presentations will be used to disseminate key scientific findings and promote the general advancement of the two fields studied.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Our approach is at the forefront of scientific and drug development in the field of anti-cancer microbial therapies and the data generated in this project have the potential to influence the progress and direction of the entire field. Such data will advance the scientific community's knowledge of how the immune system fights tumours and how this can be helped by bacteria.

Selection and characterisation of new medicine and vaccine vaccine candidates will lead to their further development (e.g. in clinical trials) and could potentially lead to new vaccines and anti-cancer therapies being introduced to the market. These products have the potential to prevent disease in or cure a significant number of people across the world. In particular, some of the vaccines we are developing are aimed at fighting infections in low/medium income countries and are poised to help tackle global health issues.

How will you maximise the outputs of your work?

This work will provide data regarding the safety and potential efficacy of new vaccines and anti-cancer therapies. The studies will result in knowledge and hypothesis generation in relation to anti-cancer therapies and will drive the development of the microbial therapeutics field.

Data and knowledge from this project will be shared with the wider scientific community by the means of scientific publications, conference presentations and patent applications.

We will be pursuing scientific collaborations with academic partners.



Species and numbers of animals expected to be used

- Mice: We expect to use around 9200 animals over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Most animals used in this project will receive one of the new medicine candidates. These can be vaccine candidates tested for their ability to elicit responses likely to provide protection against infectious disease, or the candidates can be novel anti-cancer medicines evaluated for their ability to inhibit tumour growth. Some animals will in addition undergo procedures aimed at data gathering, such as imaging. All animals will be closely monitored for weight loss, behaviour changes and clinical signs during treatment periods.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

As we try to identify and develop new medicines for cancer treatment and new vaccines for infectious diseases, studies conducted under this licence may induce some adverse effects in some of the animals. Typical adverse effects include changes in appearance, for example ruffled fur, or changes in behaviour, e.g. the animals may become subdued. Other effects may include reduction in body weight and/or reduced eating. The larger proportion of animals used in these studies will, however, not experience any noticeable 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 species)?

For the majority of animals the severity level will be mild. However, as stated above, in some studies the animals may experience some adverse effects, but these would only cause the animal a moderate level of distress which will in most cases be transient. At the end of the study, the animals will be humanely killed. After the animals are killed samples of body tissues are sent to laboratories for close examination to give more information about the biology, mechanisms of action and overall effects of the potential new medicines.

What will happen to the animals at the end of the study?

- Killed

Replacement



State what non-animal alternatives are available in this field, which alternatives you have 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 mechanisms of action and assessing the effectiveness of potential new medicines requires the presence of a fully developed and functional immune system. Currently this cannot be reproduced outside of a living organism. Indeed, the assessment of the anti-tumour efficacy of anti-tumour candidates cannot be efficiently modelled in vitro due to the complexities of the tumour microenvironment that cannot be fully replicated in a laboratory setting. Similarly, for the evaluation of new vaccines' ability to induce good protection against infection, a fully functional immune system is required.

What was your strategy for searching for non-animal alternatives?

We will aim to reduce the number of animals used in candidate testing by substituting challenge models to show efficacy of vaccination with in vitro readouts, such as neutralising antibodies assays. We will also minimise numbers of candidates to test in vivo by implementing rigorous in vitro screening candidates with high bars for in vivo translation.

We will use cell culture techniques to identify most promising anti-tumour candidates. We will also work on development of innovative cell culture techniques (such as 3D culture) to further replace in vivo screening.

Why were they not suitable?

Improved in vitro screening techniques will reduce numbers of animals used by reducing the numbers of candidates that qualify for in vivo screening and will at the same time improve the quality of candidates brought forward for in vivo screening.

However, there is a point in biological research when in vitro experiments cannot provide all the necessary conditions to further research. In vitro models can mimic certain aspects of the of disease. They cannot, however, reproduce the complex interactions between different cells and mediators or reproduce the functional changes that occur as part of the ongoing disease process. The scientific community has established extensive in vitro systems to confirm the existence of a particular mechanism. But it is only with in vivo experiments that we can establish whether new medicines targeting such pathways will be efficacious and safe.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 typical experiment may include up to 60 mice and we might run approximately 150 studies over 5 years testing new medicine candidates alone or in combination. Thus, the expected number of animals to be used under this licence is estimated at 9200.

We will use power calculations to determine the minimum numbers of mice required to demonstrate a phenotypic effect, e.g. tumour growth inhibition or immune response generation. We will consider randomised block design for our studies where possible to further limit the number of animals used. All models used will be assessed such that we shall employ the minimum severity of disease (e.g. tumour burden) required to show an effect.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

To ensure we use minimal number of animals required to obtain meaningful and relevant data, we have extensively consulted available literature, attended experimental design and statistical courses, discussed with statisticians and NC3R staff and tested online tools provided by the NC3R.

What other measures apart from good experimental design will you use to minimise numbers?

In many cases, the numbers of animals required will be reduced by longitudinal measurement of responses, e.g. by serial blood analysis for antibody response or by optimised protocols for intravital imaging. We have developed tools that will allow us to study the biodistribution of our candidates longitudinally in a single cohort. Hence, effects of new medicines may be measured after one, two or more doses in the same group of 10 mice, rather than having 10 mice sacrificed after each dose. Such longitudinal usage provides essential information on the biology and efficacy of the candidate while at the same time reducing the number of animals required to obtain such information.

Unnecessary variation in the animal cohorts that could result in requiring more mice will be minimised by use of gender and age-matched controls housed under identical conditions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The mouse is the ideal organism for our investigations for three main reasons. Firstly, there is lots of historical data available for hypothesis-building and predictions.

Secondly, there is an immense spectrum of well-characterised models developed over the years to cause minimal distress and suffering while providing meaningful data. Thirdly and in line with the previous point, development of highly effective, reproducible techniques for



longitudinal monitoring and innovative techniques focused on animal welfare provide the best setting for the purposes of this project.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The human immune system is intricately complex and modelling it for assessment of new medicines requires models in vertebrate animals whose immune systems have been studied and can be, to a good degree, compared to human. Mice are the lowest vertebrate group on which plethora of reliable information on the function of the immune system is available and where well characterised and minimal severity anti-cancer models have been developed.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

To minimise discomfort of repeated procedures such as anaesthesia, we will combine treatments under a single anaesthetic event wherever possible. The anaesthesia will preferably entail the use of inhalation agents whenever possible. Least invasive route of substance administration and needle gauge will be used where possible.

Negative control groups (baseline groups) will be minimised whenever statistically feasible.

Expertise at the Designated Site further enhances animal welfare, by providing close collaboration with dedicated animal care staff and veterinary consultants, and ready access to highly skilled advice.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow the NC3Rs guidelines on the "Responsibility in the use of animals in bioscience research" and consult all the relevant references listed therein. (Reference: NC3Rs/BBSRC/Defra/MRC/NERC/Royal Society/Wellcome Trust (2019) Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. London: NC3Rs.)

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will continuously monitor publications and the NC3Rs website for new and alternative models that could be implemented as part of this project. In addition, articles on advances in the 3Rs are regularly published on the REDACTED internal REDACTED Users News Forum and other relevant information is circulated by AWERB. Whenever possible we will implement these refinements into our studies.

Explain the choice of species and the related life stages

The adult mouse is the ideal organism for these investigations for three main reasons.

First, it is the long-standing choice of immunologists with lots of historical data available for hypothesis- building and predictions and it has successfully identified the key operating



principles of the immune system on which major clinical advances are based, e.g. the successful introduction of checkpoint inhibitors for cancer immunotherapy.

Second, and reflecting the first point, there is an immense spectrum of well-characterised models and reagents available permitting the thorough, incisive, and comprehensive obtainment of information from the experiments undertaken.

Third, there has been a development of highly effective, reproducible techniques for longitudinal monitoring, e.g. in vivo imaging, thereby reducing the numbers of animals required for parallel, staged analysis. Such innovative techniques are also focused on animal welfare, e.g. better imaging stations requiring shorter times under anaesthesia.



118. NF-κB and its role in cancer, inflammation and replication stress

Project duration

5 years 0 months

Project purpose

None selected

Key words

No answer provided

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

Cancer is now the major cause of death from disease in all age groups in the United Kingdom with approximately 300,000 new cases and 150,000 deaths each year. The lifetime risk of developing cancer is currently 1:3 and this is expected to rise towards 1:2 as life expectancies increase. Research into the onset and progression of cancer and chronic inflammatory diseases is currently being widely studied and it has been accepted that a family of proteins, called the nuclear factor-kappa B (NF-κB) family, has important roles in both.

Previous experiments from our laboratory have shown that the NF-κB family of proteins are important for the development of tumours. This is very complex, and as yet far from being fully understood. The NF-κB family of proteins are known to turn on and off genes in the cell, but we are unsure about which of these genes are turned on or off by NF-κB in cancer, or indeed in inflammation, which if is not resolved, can lead to cancer. The objective of this project is to understand the signalling events, and by this, we mean understand which components of the cell talk to and interact with one another as a result of the activation of the NF-κB proteins, which in turn are promoting and driving the onset and development of inflammation and cancer. We hope to therefore identify new mechanisms and genes associated between NF-κB and cancer, and use these to develop new targeted treatments for the disease.

The NF-κB family of proteins have well studied roles in inflammation, however, when inflammation is not resolved, or is not controlled correctly by the cell, this can lead to cancer. We will use animal models, to determine how inflammation and cancer are inter-



linked, and importantly the role of the NF- κ B family of proteins here. Once again, we will assess changes in the genes NF- κ B is controlling in this context.

We aim to use animal models of both cancer and inflammation alongside animals with known genetic changes to NF- κ B to gain further insight into the mechanisms by which NF- κ B acts during the onset and progression of cancer and inflammation. By understanding these biological processes, we hope to identify and test new therapeutics 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.

What are the potential benefits that will derive from this project?

Improved understanding at the level of cells and molecules of how organ inflammation and cancer occurs, not only advances science but helps identify new drug targets and medicines for pre-clinical testing and drug discovery.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

We will only use mice in this project of work, and expect to use approximately 15000 animals over a five year period. The animals will either be bred in-house or purchased from an authorised supplier.

This programme of work requires this number of animals as some of the colonies of mice have complex breeding requirements. For example, the mice that are genetically altered to develop blood cancer can only be successfully bred using males. This is because the females may not be well enough, or live long enough to wean their young. We therefore never use these females as breeding animals. They can be used experimentally but not mothers. Only male animals carrying this genetic mutation are used to provide future offspring carrying the mutation for both breeding (male mice) and experimental (male and female) animals. The way these mice are genetically modified to develop blood cancer, means that only one mouse in every four that will carry the correct genetic mutation causing the cancer. For every four mice we breed, only one will go on to develop blood cancer. Although some of the mice that do not carry the genetic mutation, will be used as control animals (i.e. ones carrying no mutations at all) for comparisons, or used as breeders with the male blood cancer mice mentioned above, this does further increase the numbers we need to breed .

Another colony which are genetically altered to express only one copy of the NF- κ B regulated gene Claspin, which is needed by the cell to respond to DNA damage and stress, also require more complex breeding strategies. The females within this colony have reproductive issues, and take much longer, or fail completely, to have successful pregnancies. This is something we will investigate further as part of this programme of work, but also requires us to breed more of these animals than a standard genetically modified colony in which the animals are fertile, as many cannot reproduce, and our aim is to determine why these animals have impaired reproductive function within these studies.



Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Procedures include administration of new and existing therapeutic agents, surgical interventions (removal of part of the liver to mimic liver removal in patients which is known to cause damage in people) and implantation of tumour cells. The following protocols listed on this project licence are classed as 'moderate,' and alongside a description of each there are adverse effects are discussed:

The breeding of genetically modified animals, within this project includes animals that develop tumours. Hence, adverse effects include tumour associated weight loss, inactivity or hunched posture.

Studies that are used to induce either short term or long term fibrosis of the liver could potentially result in liver failure, jaundice or dehydration, although this is very rare.

The adverse effects associated with the method used to chemically induce liver cancer include tumour associated weight loss, liver failure or inactivity.

The resection of a portion of the liver by surgical methods is associated with the liver adverse effects above (liver failure and jaundice) alongside post surgical pain, bleeding or wound breakdown.

The adverse effects of ageing mice or determining how a new genetic mutation affects an animal include; arthritis, poor coat conditions, overgrown teeth or fur loss.

Protocols which induce tumours either from a mouse or human cell background have the potential adverse effects of tumour associated weightloss, hunched posture or inactivity.

Studies which test the properties of how well new or existing treatments are tolerated in animals could result in a drop in an animal's blood pressure, a drop in body temperature or making an animal feel drowsy.

Altering the diet of mice can have different effects depending on what type of modifications are made.

For example, a high fat diet can result in obesity and an increased susceptibility to fatty liver disease. Whereas another diet used within this project can result in gradual weightloss, over grooming and poor coat conditions.

Two further two protocols on this licence are classed as 'mild' and these include taking of a small piece of ear tissue (akin to having an ear piercing in a human) in order to make cells, or stimulating the reproductive organs of the animals to assess function. Both could be associated with transient discomfort from an injection, for example. This discomfort can be minimized by ensuring staff are well trained in handling and restraint of the animals.



Animals will be monitored daily either by highly-trained animal technicians. This includes inspecting each individual cage and the animals to ensure they have a healthy body and coat condition, normal posture and that they are active and interacting with their cage mates. Alongside, this the technicians ensure the animals have sufficient access to food, water and warm bedding materials. In addition to this, animals that are used on protocols, will be additionally monitored by members of the research group at least three times each week. This will include the assessment of bodyweight, condition, and also food and water consumption. We have shown that, in the model of blood cancer, that mice tend to reduce their food and water consumption before any other overt signs of disease are apparent. If at any point an animal is found to cause a concern outside the expected range of adverse effects, advice from the Named Animal Care and Welfare Officer and Named Veterinary Surgeon will be sought, and appropriate treatment will be given. If required, and advised the animal may be humanely killed.

Furthermore, to ensure protocols involve the least suffering we will make maximum use of all information to minimize any potential adverse events. All studies will be in accordance with the guidelines set out in Workman P et al. (2010). Guidelines for the welfare and use of animals in cancer research. Br J Cancer. 102:1555-77

Animals will be humanely killed at the end of protocols within this project.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Due to the availability of animals with relevant genetic make-up combined with the fact that both humans and rodents have very similar NF- κ B proteins, mice are the only suitable species of animal to perform these studies.

We are accumulating large banks of frozen and paraffin embedded tissue from previous experiments.

These are used wherever possible to minimise the further use of animals. Wherever possible we will use human tissue or cells. We have ethical approval to access and use human lymphoma tumour samples that were taken, and paraffin-embedded, at time of surgical resection. This also makes it much more likely that our findings will have direct applications to help treat patients in the future.

Reduction

Explain how you will assure the use of minimum numbers of animals.

To reduce animal use we will use power analysis or search the current literature to determine the minimum number of experimental animals.

We are able to grow tumours cells from our mouse models of blood cancer in tissue culture, hence reducing the number of animals we will need to use in some further studies to assess the role of NF- κ B in blood cancer. These cells can also be transplanted into multiple recipients to reduce the numbers of genetically altered mice that we need to breed.



We are also developing a novel method of tracking chemically induced liver cancer using imaging techniques which has shown to give robust results using far less animals.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Our mouse model of blood cancer is widely studied in the field and are genetically altered to model human disease in the best way possible. It has been consistently shown to reflect human disease onset and progression and provide a robust, sophisticated model for our proposed project of work.

The model of chemically induced liver cancer mice we have used is known to be less severe than other reported models that produce liver cancer more rapidly but have a higher mortality rate.

Any pain relief and supportive care will be administered as required for the species and model. For example, analgesics in order to minimise pain.



119. Noninvasive Ultrasound for Therapy and Diagnosis

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants.

Key words

No answer provided

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The overarching aims of our research project are:

To understand the mechanisms of how ultrasound with or without acoustically active particles (e.g., microbubbles) interacts with biological tissue.

To design, create, optimise, and characterise novel ultrasound technologies, acoustic particles, therapeutic agents, diagnostic agents, and procedures for the treatment and diagnosis of diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

The use of ultrasound and acoustic particles in the clinic has continued to increase over the last several decades both as diagnostic and therapeutic technologies. Ultrasound applications are expanding from their traditional use for imaging tissue structure towards high frame rate (>2 kHz) imaging, imaging tissue function and other capabilities. Meanwhile, therapeutic ultrasound is experiencing an even greater revolution of capabilities. Therapeutic ultrasound has been shown to dissolve clots to treat stroke, open



capillaries to enhance drug delivery and a wide range of other therapeutic bioeffects, many of which we aim to understand and control here. A UK “Report of the Independent Advisory Group on Non-ionising Radiation” (Health Protection Agency, 2008) highlighted the necessity to address (1) the knowledge gap of how ultrasound with or without acoustic particles can produce different effects and (2) how these effects can be controlled so that the desired objective can be produced while avoiding adverse effects in patients. Our aims are to identify the mechanism of these interactions, to be able to predict and therefore control the beneficial biologic effect and to be able to use this technology to the development of basic research and the treatment of human diseases.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

5000 mice.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Animals inoculated with tumourigenic compounds are likely to develop tumours. We will not allow the tumour to reach a size that affects the overall health of the animal. As the tumour grows, potential adverse effects, such as tumour ulceration and a hindrance to ambulate, will be monitored closely. If signs of ill health are noticed a Named Animal Care & Welfare Officer (NACWO) and/or Named Veterinary Surgeon (NVS) will be notified and the animal will be monitored as appropriate. Palliative care may be provided as suitable.

We will follow standard guidelines (LASA) when collecting blood samples. The amount of blood sampled will not exceed an amount that will affect the overall health of the animal. Following the collection of blood samples, the puncture site will be cleaned and monitored in a manner similar to what we experience at the hospital.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

The ultrasound technology developed here aims to perform non-invasive diagnostic and therapeutic procedures on biological tissue. One of the main challenges with studying these phenomena and methods is that ultrasound interactions occur at the micron-, and nano-scale 3-dimensionally. Current 3D tissue culture models are unable to model complex and clinically relevant microenvironments.

Also, ultrasound reflects strongly to material with a high acoustic impedance mismatch, such as glass and hard plastic materials, which are commonly used for tissue culture setups. These mismatches alter pressure waveforms in tissue culture setups that may



never exist in vivo. Therefore, only a higher mammalian organism can be used to reproduce many of the important 3D tissue microenvironments.

Reduction

Explain how you will assure the use of minimum numbers of animals.

We will reduce the number of animals used in our experiments by using alternative methods:

Theory, computer simulations, and experiments on tissue-mimicking material will be extensively used prior to in vivo experiments to select, validate and optimise the biological model and the ultrasound parameters.

Long studies using the same mouse that do not significantly increase the severity of the protocol allow for overall reduced number of animals to be used.

During the experimental design stage of the studies, we will ensure that the data obtained throughout each stage will provide as clear of a conclusion as possible while using as low a number of animals as possible.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Mice were chosen because they are the lowest animals on the evolutionary tree for which many suitable models of diseases (e.g., cancer) are available.

Subcutaneous cancer tumours will be visualised by eye and measured by callipers. Tumour size will be monitored to ensure the animals do not experience any discomfort. The sizes of tumours used under this licence are unlikely to affect the health of the animals. Cancers not observable by the naked eye will be monitored by imaging when appropriate.

Adverse effects due to the administration of therapeutic or diagnostic agents will be monitored in accordance with NCRI guidelines for the Welfare and use of animals in cancer research. LASA Good Practice Guidelines on the Administration of Substances will also be followed.

When we want to use ultrasound in a way that does not affect the biological tissue, ultrasound exposure conditions will be selected to remain within clinically defined limits. However, the purpose of many of our applications is to understand and control how ultrasound alters the biological tissue structure and function to optimise beneficial effects while minimising adverse effects. As a result, some of our investigations will use exposure conditions that will alter the tissue. The following principles will be followed in order to refine our exposure conditions:

Reduction of the region of ultrasound exposure by careful positioning of the ultrasound transducer(s) or by focussing ultrasound to a small target region.



Home Office

Limiting exposure parameters that are not contributing to the advancement of our understanding.



120. Nutrition of poultry

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.
 - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes.
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants.

Key words

No answer provided

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The project aims to establish nutritional strategies that promote growth, production and gut health in poultry, while reducing competition for resources for food and feed. To reduce nutrient excretion in the environment and its negative impacts on animal production, feedstuff's (including unconventional feedstuffs) will be tested for an accurate estimation of energy and nutrient utilisation. Non antibiotic feed additives will also be tested using different dietary interventions to address global food safety and public health issues linked with antibiotic resistance and ability of poultry species to resist infection.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

The knowledge attained from this project will be used by poultry feed additive and feed manufacturing companies to alter the formulation of feed to meet the animal requirements and reduce nutrient excretion in the environment (for example the use of phytase to reduce phosphorus excretion to water bodies or in the manure). Feed costs account for nearly 70-80% of the total costs in poultry production. In many cases, this may be out of



reach for smallholder poultry farmers who lack economies of scale and access to credit. Therefore, identifying the energy and nutrient profile of feedstuffs (conventional and unconventional) will help in long term to provide alternative feed resources and reduce competition between man and animal for food resources.

This work is of special interest to researchers (worldwide), food retail industry and poultry producers as it will increase our knowledge on the host response to diseases and will help to develop alternatives to antimicrobial growth promoters (feed additives) to reduce carriage of food borne diseases and identify interventions that can influence the ability of poultry species to resist infection and production of safe/healthy poultry products for human consumption.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Species to use are broilers and turkey. Nutrition and efficacy studies: 86,000 (13,440 layers, 63,560 broilers and 9,000 turkeys); Evaluation of feed ingredients – precision feeding: 900 (450 broilers or broiler breeder and 450 adult cockerels); Nutritional evaluation of diets: 11,000 (8,840 broilers and 2,160 turkeys); Standardised and true digestibility studies – 5,000 (4000 broilers, 1000 turkeys), Nutrition and gut health studies – 25,000 (20,000 broilers and 5,000 turkeys).

The calculation of approximate number of animals required in each protocol is based on the new larger poultry holding capacity of the institute and type of studies that may be required over next 5 years. The calculation also takes into account the number of studies conducted and the animals used in each study over last 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

In feeding studies, some of the birds in the project will receive diets that are not meeting nutrient requirement and such birds will be expected to have a 10 to 15% of body weight loss compared to aged matched control group.

In precision feeding, if any animal showed sign of resistance during gavaging the procedure will be immediately stopped for that specific bird and the amount yet to be fed will be noted and discarded.

In case of laying hens, if feather pecking becomes a problem we will consider beak trimming (if not already beak trimmed or badly trimmed). Veterinary advice will be sought if any bird showed gentle pecking injury. To prevent further pecking affected birds will be treated with anti-peck spray. If any bird shows signs of aggressive feather pecking (forceful pecking of featherless skin followed by bleeding) than appropriate action will be taken to minimise the suffering of the animal and if recovery is not expected within short time frame



(24-48 hours) the animal will be culled. If 2 or more birds, or 10%, whichever is greater, have to be culled or die due to injurious pecking, then that entire replicate will be culled.

Any bird developing severe swelling, ulcers or scabs on footpad or hock lesions will be humanly killed. In case of blood sampling, If any animal develops adverse effects, it will be culled. In establishing the standardised and true digestibility for nitrogen, amino acids and minerals using purified and semi-purified diets, some of the birds in the experiment will be provided with diets with very little or even no protein or mineral for a very short period, not exceeding 7 days. Any bird that exceeds 20% of weight loss compared to the starting weight will be humanly culled.

In case of *Campylobacter* challenge studies, birds will be screened for *Campylobacter* before recruitment onto the study and the trial will not commence if birds are found positive for *Campylobacter*. Birds exposed to *Emieria spp.* that develop clinical disease symptoms (high percentage of visibly sick birds, severe bloody diarrhoea etc.) will be removed from the experiment and culled. If more than 30% of birds in pen are unintentionally becoming clinical, then whole pen will be culled i.e 3 birds in pen of typical 10).

At the end of the experiments, some of the birds will be euthanised using humane methods. Some of the procedures do not require euthanasia of birds as part of the experiment and such birds may be rehomed to backyard flocks.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Studies that require effect of treatments on growth can't be done in non-animal substitute. Digestibility studies are usually preceded by in vitro proof of concepts but ultimately because the feed additives will be incorporated to feed for actual animals, it is a requirement that such products are fed to animals.

A minimum of 3 independent in vivo studies showing significant effects are required by European Food Safety Agency (EFSA) to demonstrate efficacy of the feed additive for the relevant target species/categories (Regulations (EC) No 429/2008).

Reduction

Explain how you will assure the use of minimum numbers of animals.

The number of replications and animal per replications are determined based on statistical analyses and previous experience. Each experiment is individually set up to maximise ability to test for treatment effect combined with every effort to use the minimum number of animals. Experimental protocols are reviewed before each experiment to ensure that animals are not used unnecessarily. In addition, there are monitoring exercises after each experiment to see what lessons are learnt. The 3Rs is part of the monitoring exercises. Part of the review of the experiment include the input of expert statisticians.

Sample size calculations will be based on the magnitude of the the effect that the study is designed to test, its variability, an adequate statistical power and the confidence level as required by European Food Standard Authority (EFSA) for assessment of the efficacy of feed additives (EFSA Journal 2018:



<https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2018.5274>) or safety of feed additives for target species (EFSA Journal 2017:

<https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2017.5021>)

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The objective of this project is to provide information that is relevant to the poultry industry, and hence poultry species are the choice of animals for the project. There are instances where nutritional effects studied are applicable across species (i.e. what is studied in broilers may be applicable to turkey) and in such cases, studies are not repeated for all the poultry species. Most of the protocols in this project are mild in severity level except protocol 4 & 5 which are moderate. Birds used in this project will be monitored on a daily basis to ensure that bird's well-being is not compromised. There is also on-site specialist (avian) veterinary support who helps ensure birds well-being is maintained. In disease challenge studies, only use sub-clinical infections models are used. All studies will be assessed pre and post conduct by the institute's AWERB whose primary function is to assess the ethical and scientific validity of the study proposed and , post study, the efficacy of the study and lessons learned. The evaluation of the study post conduct allows us to further refine our procedures from time to time.





121. Opioid receptors in depression and anxiety

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

No answer provided

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

Depression and related illnesses affect 1 in 6 people in the UK. Depression is the leading cause of disability worldwide, and is a major contributor to the overall global burden of disease. Almost twice as many women as men are diagnosed with depression. Stress is a major risk factor for developing depression. The medicines available for treating depression are limited because less than half of all depressed people get completely well after taking them. These medicines are also slow to work (weeks to months), may have side effects and you can't just stop taking them suddenly as that can make the symptoms worse. We need new and better medicines for depression.

The aim of this project is to learn more about how opioid receptors in the brain may be involved in the response to stress and how we can use this information to design new medicines for depression. Opioids are chemicals found in brain cells that signal to other brain cells using different types of opioid receptor. The opioids are named endorphins, enkephalins and dynorphins which work through switching on mu-opioid receptors, delta-opioid receptors and kappa-opioid receptors, respectively. We're investigating how these different opioid receptors contribute to the brain's response to stress and how we can target them to make new antidepressant 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.

What are the potential benefits that will derive from this project?



Understanding how the different opioids and their receptors help the brain respond to stress will allow us to design new medicines for depression. Using this knowledge we can design opioid medicines that target specific receptors and these may, in the long term, be useful in the treatment of depression.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

We estimate that we will use 6,450 animals over the lifetime of the project (5 years): 1000 genetically altered mice, 5300 standard laboratory mice and 150 laboratory rats. Both adult and juvenile, male and female animals will be used.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Genetically altered mice will be used in this project (15% of animals used). Being able to interfere with opioid receptor signalling genetically will help us define the roles of individual receptors. The genetic alterations used for this project do not have a big effect on the mouse. For example, they don't change the way a mouse looks or moves or feeds so can be said to be 'not harmful'.

Around 7% of animals will be humanely killed, under general anaesthesia, to provide brain tissue for analysis after death. This results in no more harm than when a vet puts down an animal.

Another 7% of animals will undergo these same procedures but will have injections of drugs to interfere with the opioid receptor signalling pathway so we can identify the involvement of specific signalling molecules. Drugs will be used at doses that produce the desired effect, have no toxicity and injected in small volumes by trained and competent experimenters. Under these circumstances, the harms are expected to be minor stress and discomfort from the injections and the animals show a rapid return to normal behaviour.

Around 31% of animals will be used to investigate how the brain responds to stress. Chronic stress is a risk factor for the development of depression. In order to understand the role of opioids in response to stress, we need to study the adaptations to repeated stress. The stress procedure in mice has to be one that can produce significant physiological and behavioural change consistently. Repeated stress is likely to cause moderate impairment of the well-being of the animals. We will develop a paradigm that achieves our objectives while limiting the harms to the mice. Limits are placed on the duration, frequency and intensity of the stress to reduce the harms to animals. Monitoring of behaviour and appearance (body weight, coat appearance, movement, posture) on a daily basis ensures animal welfare is maintained and humane endpoints are in place.



The behavioural adaptation to repeated stress will be investigated using non-invasive mouse behaviours in a range of different tasks. Using multiple tests of different dimensions of behaviour increase confidence in the interpretation of the data. Typically, tests involve exploration of a novel environment such as a maze or self-grooming in response to sugar sprayed onto their coats. These behavioural tests cause only minor and transient discomfort induced by the test itself and also handling or drug injection. Animals are observed throughout the tasks and monitored on return to the home cage to ensure there is normal home cage behaviour. Limits are placed on the duration and frequency of testing to reduce the harms to the animals. However, when coupled with repeated stress procedures, the cumulative experience of each individual step is likely to cause moderate impairment of well-being.

Testing new potential opioid medicines in a mouse for the first time (4% of animals used) can cause unexpected effects and, very rarely, death. Before testing in a mouse, we do experiments with the new compound using cultured cells and isolated tissues to learn as much about the dosing and likely effects of the medicine before we use it in a mouse. When we test for the first time, we use only two mice and monitor their behaviour – posture, walking, coat state, breathing - carefully using a score sheet before proceeding to use further animals. If animals are showing abnormal behaviours they will be killed humanely and doses lowered before proceeding to test two further animals. We also need to demonstrate that the new opioid compound actually targets the opioid receptors in the predicted way (4% of animals used). This can be done by taking advantage of opioid pain-relief properties to assess the drug profile. Mice will have their tail tip dipped in warm water and experience mild and transient pain that they can escape from. This helps us work out how much of the new compound to use and what effects it is likely to have. Limits are placed on the intensity, duration and frequency of testing to reduce harms. We then use the fewest animals possible to find the right amount of the new compound to establish the potential antidepressant- and/or anxiolytic-like properties in a variety of non-invasive behavioural tasks (31% of animals).

Using multiple tests of different dimensions of behaviour increase confidence in the interpretation of the data. Typically, tests involve exploration of a novel environment such as a maze or self-grooming in response to sugar sprayed onto their coats. Additionally, the forced swim test is used. In this test, mice are gently placed in a large cylinder of water for 6 minutes. Mice engage in active escape-related behaviours and periods of immobility. Mice are naturally buoyant and 'immobility' is a floating behaviour where minimal effort is used to keep their heads above water. The time spent immobile is reduced by antidepressant drug treatments and can therefore be used as a measure of antidepressant potential for new compounds. These behavioural tests cause only minor and transient discomfort induced by the test itself and also handling or drug injection. Animals are observed throughout the tasks and monitored on return to the home cage to ensure there is normal home cage behaviour. Limits are placed on the duration and frequency of testing to reduce the harms to the animals.

At the end of the experiments all animals are killed humanely. Brain tissues may then be removed for further investigation e.g. to see whether there are changes in gene expression in response to stress or drug treatment.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.



The way the brain responds to stress involves a complex network of different brain regions that can only be studied in a whole animal. Non-mammalian species do not have the same brain networks as mammals and so cannot be used. Human brain imaging studies could be used to study individual opioid receptors but there are no tools to be able to do this at present. New technologies using human stem cells to create 'organ-on-a-chip' and 3D cell cultures or 'mini-brains' are emerging but do not yet produce sufficient network connectivity to mimic an adult brain. It is also not possible to trial new potential opioid medicines in humans without first investigating their effects in an animal. Non-animal alternatives like cell cultures and isolated tissues are a vital part of this project. For new opioid compounds we can work out the likely effects and how much we need of these compounds before testing in animals.

Reduction

Explain how you will assure the use of minimum numbers of animals.

The minimum number of animals is determined using statistical methods. We have extensive experience with these techniques to ensure the robustness of statistical analysis.

Good experimental design to reduce variability is essential to maximise the data obtained from the minimum number of animals. Behavioural measures are carefully controlled including variables such as age, sex and strain; husbandry and handling; laboratory environment (light, noise) which also limits the stress to the animals.

We also try to get multiple different types of data from each mouse where this does not increase the harms. For example, we can study behaviour in the mouse, collect blood samples to tell us about hormonal stress responses and then, at the end of the experiment, take brain tissues to look at gene expression.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Rodents are the lowest mammalian species that are appropriate for this work. There is extensive scientific literature on the brain circuits, nerve cell function and opioid receptors in mice and rats. Some studies, where urine is to be collected, are best performed in rats to obtain sufficient quantities of urine to have robust and reliable data.

The majority of procedures carried out in this project cause only transient and minor discomfort to the animals. Limits are placed on the duration, intensity and frequency of procedures to reduce the potential harms to the animals. Behavioural tests used are non-invasive. Typically, tests involve exploration of a novel environment such as a maze or self-grooming in response to sugar sprayed onto their coats. These behavioural tests cause only minor and transient discomfort induced by the test itself and also handling or drug injection. The impact of these is reduced by good technique, for example, handling by gentle cupping of mice and competent injection technique.

Animals are observed throughout the tasks and monitored on return to the homecage to ensure there is a rapid return to normal homecage behaviour. Limits are placed on the duration and frequency of testing to reduce the harms to the animals.



Repeated stress is likely to cause moderate impairment of the well-being of the animals. We will use a minimally effective paradigm that achieves our scientific objectives while limiting the harms to the mice.

Limits are placed on the duration, frequency and intensity of the stress to reduce the harms to animals. Repeated stress can cause loss of body weight and hormonal stress responses. The harms to the animals are reduced by careful daily monitoring of home cage behaviours, and absolute limits set on the amount of weight loss, to define humane endpoints.

For blood sampling, we use a refined procedure that involves gentle cupping as a restraint, no anaesthesia and no needles where blood can be wasted. Limits are placed on the frequency and volume of blood that can be collected to avoid a significant drop in total blood volume that could affect blood pressure and circulation to the body. Repeated sampling from the same mouse within these limits minimizes the number of animals used overall, and improves the scientific variability of the data, as each mouse can be its own control.

For new opioid compounds, a small number of animals are used to establish a safe and effective dose before screening for antidepressant or anxiolytic properties in a range of behavioural tasks. Data obtained from cell cultures and isolated tissues informs the choice of initial dosing and animals are carefully monitored to minimise harms.





122. Opportunistic pathogens

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.

Key words

No answer provided

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The overall scientific objectives of the project are to determine the reasons for which opportunistic bacteria and fungi are able to cause infections in patients with chronic respiratory disease or immunosuppression/deficiency. For instance, there are around 10 million individuals globally with respiratory forms of aspergillosis. The main individuals at risk are those with chronic lung diseases such as asthma or smoking-related lung damage, as well as individuals with weakened immune systems due to cancer treatments or because they have had organ transplants. This will enable identification of the primary deficiencies in patient immunity, and which immune cells are primarily affected. The altered immune response may also lead to the emergence of bacterial or fungal behaviours that enable infection to occur when host immune responses are suppressed. The development of new clinically-relevant animal models of infection will further enable the identification of the functional basis for any such factors. This will enable the development of novel diagnostic tests to identify at risk patient groups and enable the development of novel treatment to boost the immune system response when infection occurs

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?



The research seeks to understand the key factors within both the pathogen and the patients immune system that lead to an infection occurring. This will allow us to develop new diagnostic tests to identify that people who are at risk of or likely to have a serious infection. We will also seek to develop new targeted treatments of the immune system or new antibiotics that can improve the chances that a person will survive the infection or get rid of the infection.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Approximately 9000 mice (maximum) and approximately 10000 (maximum) zebrafish will be used over a 5 year period.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

We are breeding adult zebrafish to generate larvae (juvenile forms) to use in our research. Occasionally adults need to be humanely killed due to old age or illness. Where zebrafish adults are used for infection studies, the effects are moderate and at the end of the study they will be humanely killed. Expected adverse effects include: abnormal swimming behaviour, separation from the shoal, abnormal scale position, abdominal swelling, abnormal skin outgrowth. Fish will be regularly monitored for such effects and killed using a humane method if such effects are seen.

The mice may receive immunosuppressive drugs and will be infected with either bacteria or fungi. This will lead to establishment of infections, which may cause the animals to develop signs of infection. This may lead to increased breathing rate, fevers or hunched posture. This is not expected for the majority of infections. The level of severity is categorised as moderate. After infection animals may also undergo administration of pharmacological agents, which may cause the animals to become distressed. The level of severity is categorised as moderate. At the end of the experiments the animals will be humanely killed. Suffering will be minimised as all infections will be undertaken under general anaesthesia and in addition 20% weight loss has been established as a surrogate marker for death previously (and which point mice will be humanely killed).

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

The immune response to an infection is controlled and influenced by multiple factors, including



how aggressive the microbe is and the the infected persons genetic make-up. Teasing out the multiple factors that contribute to infection in humans is extremely complex as many of the variables remain unknown.

Using animal models to study infection, the researcher can focus on individual variables during the infection process in order to establish their relative importance.

We use zebrafish in preference to mice for most experiments because

They develop fast

They are vertebrates and have a fully developed immune system (larvae have innate immunity only, adults can be used to study role of adaptive immunity)

The eggs / larvae are clear and easily observed and manipulated at the microscope.

Where we use mice, this is generally to enable us to study the complicated immune responses that occur in the lung, as zebrafish do not have lungs. In addition, there are some parts of the immune system in humans that are not well represented in fish but are present in mice. Also for some of our therapeutic and diagnostic development murine experimentation is a requirement for subsequent use in humans. Therefore, the overall approach will be to use zebrafish for most experiments but only use mice where necessary because zebrafish lack the relevant biology, or when confirmation of findings is required in a mammalian system. I envisage this will lead to a substantial reduction in mouse usage.

Reduction

Explain how you will assure the use of minimum numbers of animals.

All experiments will be designed so that the minimum number of animals will be used. REDACTED This way the need for repeat experiments or increased animal numbers is minimised. Breeding strategies are carried out by qualified personnel and aimed to avoid unnecessary animal generation (animal surplus). In addition, we are developing novel imaging-based models of infection that will enable further reduction of the number of animals used by allowing longitudinal studies.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Zebrafish will be used where possible because they are easily genetically modified, fast breeders and the larvae are small and transparent which make it possible to follow individual cells and their behaviour using a microscope. We will mostly use larvae, where suffering is reduced compared to adults or mice as they are less able to sense pain.

Animal suffering will be minimised by regular checking of the animals for relevant symptoms that constitute the end point of the experiment. All breeding and husbandry procedures will be performed by trained staff, and the fish monitored regularly.

Mouse models of infection offer several distinct advantages for studying infections, including ease of use, reproducibility and availability of immunosuppression regimes which



mimic patient factors of human disease. Additionally, mouse models offer the opportunity for a thorough investigation of immune responses and are amenable to novel diagnostics, which falls beyond the scope of this application but will become important in our future research and will facilitate standardisation across the present research community within the context of important novel findings. Suffering will be minimised as all infections will be undertaken under anaesthesia and in addition 20% weight loss has been established as a surrogate marker for death previously (and which point mice will be humanely killed).





123. Pancreatic Cancer - Exploring biology and options for 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

No answer provided

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 is the aim of this project?

The aim of this project is to investigate the importance of mutations and inflammatory pathways found in human pancreatic cancer in mouse models, and to characterize different subsets of pancreatic cancer caused by different mutations to understand the disease better and find specific targets for therapy. The models will then allow us to perform trials of new, potentially targeted therapies (which are specific to different mutations or pathways) in these clinically relevant mouse models of pancreatic 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?

Pancreatic cancer is the 4th commonest cause of cancer deaths in the western world, and is predicted to be the second most common cause within the next decade. Current therapies are largely ineffective, meaning that the median survival is just 6 months, and



only 6% of patients survive 5 years following diagnosis. This situation has remained virtually unchanged for the past 50 years making pancreatic cancer a huge unmet need.

A distinguishing feature of the disease is the dense microenvironment, almost like a healing wound, that surrounds and supports the tumour cells and can account for up to 90% of the tumour volume in human pancreatic cancer. This environment comprises fibroblasts (wound healing cells), pro-tumour immune cells, blood vessels, and structural proteins like collagen and fibronectin. All these components play an important role in disease progression, able to influence tumour cell proliferation, survival, metabolism, migration, immune surveillance, and response to chemotherapy. As such, it is essential to investigate these aspects of tumour biology in vivo, in spontaneous tumours with a physiological microenvironment and immune response.

What outputs do you think you will see at the end of this project?

We study the genes that go wrong in pancreatic cancer so that we can increase our understanding of how pancreatic cancer develops, progresses, and spreads to other sites in the body. We also aim to find new ways to target the disease therapeutically and understand how we might best treat patients. Our project concentrates on pancreatic cancer, but our work could apply to many cancer types. Outputs should include findings that can direct new clinical trials, and publications in peer reviewed journals.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Pancreatic cancer is the fourth commonest cause of cancer deaths in the UK. Studies from this project licence should provide insights into pancreatic cancer biology, and this will benefit the rest of the pancreatic research community in the short term. Our studies will enable us to test novel targeted anti-cancer therapies in mouse models that mimic the human disease, so longer term, we hope that our work benefits patients and their families, and the medical professionals treating this disease. We will share what we learn with the rest of the scientific community by publishing our findings in scientific journals and giving presentations at scientific conferences both in the UK and internationally. We will also communicate our findings to the public through open evenings in our Institute, and meetings with supporters, patients and advocates in our Institute and around the country.

How will you maximise the outputs of your work?

We will continue to collaborate with other groups involved in cancer research, particularly pancreatic cancer, and disseminate our knowledge (both positive and negative) to the community. New findings arising from these studies will be submitted for publication in peer reviewed journals and presented at national and international meetings.

Species and numbers of animals expected to be used

- Mice: 50,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The majority of mice in the project will not suffer any adverse effects because they will not express all the right gene mutations to develop pancreatic cancer. With regard to the mice carrying the right gene mutations, most will develop pancreatic cancer as adults and be humanely killed without additional procedures when they show symptoms of pancreatic cancer (swollen belly, weight loss around haunches).

Some mice will be used in preclinical trials. That means that they will be monitored for tumour development by imaging (PET-MRI, fluorescent imaging, but most commonly ultrasound) under anaesthesia (typically for less than 10 minutes. Some of these mice will have their tumours biopsied by keyhole surgery under anaesthesia with pain relief in their drinking water pre- and post surgery. They will then be enrolled on a preclinical trial. The route and schedule of drug administration will depend on the drug, but they might be injected with anti-cancer agents from twice daily, to weekly). They will also be imaged weekly (again usually by ultrasound). They will be humanely killed when they show symptoms of pancreatic cancer, which is usually ~3 weeks after being enrolled on trial, unless the therapy is effective, in which case time on treatment will not often be longer than 2 months.

A small number of mice will have imaging windows surgically inserted into their skin to allow fluorescent imaging of the live interactions between tumour cells and their surroundings.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Most mice will not experience any clinical signs, because they will not express all the right gene mutations to develop pancreatic cancer, however some mice will develop pancreatic cancer. Mice with pancreatic cancer appear to be a lot like humans with pancreatic cancer, in that they don't exhibit any discernible symptoms until very late in tumour progression. This means that they may exhibit mild symptoms for ~5 days, and moderate symptoms for up to 24 hours before being humanely killed. Symptoms are most often a swollen belly, and weight loss around the haunches and this could be apparent for 3-5 days. With late stage cancer, occasionally mice may develop jaundice (a yellowish hue to the skin) or may become less mobile, hunched and/or exhibit a scruffy coat (usually not more than 24 hours). At this point they will be humanely killed. When mice undergo surgical biopsy or surgical implant of tumours or imaging windows, there could be transient discomfort (~2 days), but this will be alleviated with pain relief.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

All the animals used will be mice. The expected severities are as follows:

~80% mild (those mice that do not develop pancreatic cancer or are euthanised at early time- points before displaying any symptoms)



~20% moderate (predominantly mice that develop end-stage pancreatic cancer)

What will happen to the animals at the end of the study?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Pancreatic cancer is a very complex cancer that involves a number of different cell types e.g. cancer cells, wound healing cells, immune cells, blood vessels, all within a particularly dense environment.

This environment, and all these different cell types, play a major role in tumour development, growth and spread, and stop drugs from working properly. Non-animal models cannot reproduce this situation and are not appropriate for studies to understand pancreatic cancer progression or for testing of new anti-cancer drugs.

What was your strategy for searching for non-animal alternatives?

We considered using cell line and (organoid) 3D cell culture experiments, and indeed we do use these where possible. We also examining human tissue samples where possible.

Why were they not suitable?

Cell line and organoid systems allow us to test the effects of drugs, or of deleting/activating cancer-causing genes in cells in plastic dishes but they invariably failed to deliver results in terms of predicting success in clinical trials in patients, and indeed can generate the opposite results. Indeed, many clinical trials based on results from these systems have failed, with the reasons for failure subsequently demonstrated in mouse models. (It's important to note that preventing negative clinical trials from being carried out in patients is as important as driving successful trials). On the other hand, immunotherapy, which has been a game-changer in cancer research was developed almost entirely from research in mouse models.

Cancer cells evolve and adapt to survive, and it is becoming increasingly clear that once removed from the supporting environment in which they normally grow, they 're-wire' many of their signalling pathways and no longer represent the tumour from which they came.

Archival human samples give us information about different mutations or altered signalling pathways, but they are not suitable for most work because any hypotheses are speculative without testing in 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?

Many of our strains have a predictable phenotype and this allows us to be quite accurate in the estimation of mouse numbers we need for each experiment. However, we use very complicated genetics in our models so that we need to breed a large number of mice to generate ones carrying all the genes necessary for each experiment. The numbers are based on these factors, as well as the number of genes and pathways that we plan to investigate.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The use of in vivo imaging e.g. ultrasound allows us to follow the same mouse during tumour progression and reduces the need to euthanize different mice at a number of different timepoints to assess tumour burden. Pre-treatment biopsies also allow us to reduce the number of mice used for timepoints. Thus, these approaches have been factored into experimental design.

The therapeutic agents that we will use in these studies will be screened in vitro for activity against the molecules that we want to target, and will be proven to have potential for development in the clinical setting (e.g. the target molecule is expressed in human and mouse pancreatic cancer), reducing the number of animals used in pre-clinical trials. We now have a dedicated bioinformatician within our lab who we continually consult to ensure best practice in experimental design.

What other measures apart from good experimental design will you use to minimise numbers?

Human pancreatic cancer occurs over a number of years and as a result of many mutations in cancer-related genes. To develop mouse models that mimic the disease, mice have to carry multiple mutations. In addition, some 'next generation' models allow us to target 2 different cell types (e.g. tumour cells and immune cells), or target only the tumour cells but in a sequential manner (for example to test the effects of deleting a gene from an established tumour). Thus, many of our cohorts involve breeding together of multiple genetically altered lines. Our breeding programmes are constantly optimised to generate the highest proportion of experimental mice possible, however, due to the complex genetics only a proportion of animals born will be of the desired genotype. We try to reduce the numbers of excess mice generated this way by maintaining stock animals carrying multiple mutations in a non-harmful combination, and where possible we minimize numbers of controls by extrapolating from different cohorts.

We collect tissue from all experimental mice which we share within the Institute and indeed throughout the UK. We also routinely prepare cell lines and organoids for use by our lab, but which are actually very often used by other labs in the Institute and around the UK, this reducing the number of mice used in pancreatic cancer research. In fact, we are involved in a project which brings together pre-clinical pancreatic cancer researchers from across



the UK and prevents duplication of effort while maximising the benefit of our collective learnings.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice carrying the same mutations as human pancreatic cancer, specifically within their pancreas, will be bred to show predisposition to pancreatic cancer. We might also alter genes within 'normal' cells that can help drive cancer, for example, wound-healing cells, or we may induce inflammation in the pancreas, as this is a risk factor in humans. Some mice will receive a transplant of tumour tissue or cells from mouse or human cancer (occasionally via surgery).

Mice will develop pancreatic tumours or metastases (spread in other organs) that mimic the human disease. Pancreatic cancer is a very complex cancer involving a number of different cell types, so more refined models in cells or in less sentient animals don't mimic the disease well. Therefore to be able to effectively model human pancreatic cancer and metastasis, we need to observe metastatic pancreatic tumours in the mice. Mice with early stage pancreatic cancer do not actually exhibit signs of suffering or distress, and where possible mice will be humanely culled before showing any signs of suffering, however, mice with late-stage pancreatic cancer will exhibit some clinical signs (usually a slightly swollen belly and weight loss around the haunches), and unfortunately, it is necessary to allow some mice to develop established pancreatic tumours to accurately model the clinical situation. Mice showing these signs will be humanely culled.

In some cases, to mimic clinical trials, a biopsy will be taken and then mice enrolled onto a mouse 'clinical trial', using either clinically relevant treatments, or further genetic alterations where drugs do not yet exist, combined with imaging. Where any surgery is performed some pain or discomfort is expected so this will be kept to a minimum with analgesia. We expect mice to recover normally in 2-3 days. Once on therapy, mice will be given treatment by experienced staff using the most refined techniques (single use needles and aseptic).

For surgery or for imaging, anaesthesia will be used. The body temperature of the mice under anaesthetic will be maintained using heated platforms, and normal mouse food may be supplemented with gel diet or other treats after anaesthesia.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Pancreatic cancer, particularly, is a very complex cancer that involves a number of different cell types e.g. cancer cells, immune cells, wound-healing cells, and a particularly dense 'matrix'. All of these factors combine to play a major role in tumour development, growth and spread, and resistance to anti-cancer drugs. Mouse models of cancer are widely accepted to be the most closely representative of human cancers. We use state-of-



the art genetic models to ensure that the cancer develops in the correct tissue (the pancreas), with the same mutations that are found in human pancreatic cancer. Tumours progress through the same stages of pre-cancer as in humans and also spread to the same organs. Other models cannot reproduce this situation.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Robust standard operating procedures for animal care, monitoring and minimal handling are in place. Social, environmental and behavioural enrichment are also provided. Lab members will ensure that all animals receive the highest standard of care. We have a vast amount of experience with mouse models of pancreatic cancer, and close monitoring of tumour development allows us to recognize clinical signs before the animals exhibit signs of pain. Imaging, using small animal ultrasound, also allows us to detect internal tumours at much earlier stages, so in many cases we can monitor effects of chemotherapeutic drugs in tumours before mice develop symptoms. Where required, for example pre- and post surgery, preventative medicine (anaesthesia, pain relief, heated cages) will be used.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

For all of our studies we will abide by published guidelines (NC3Rs, Workman et al, 2010 *Br. J. Cancer*), as well as local guidelines, and ensure best working practice. We will also adhere to the ARRIVE guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs website and elsewhere). In our facility, compulsory monthly user forums are also in place for all personal licence holders, and 3Rs advances are introduced here and implemented across the facility.

Explain the choice of species and the related life stages

We will be using mice that have been genetically engineered to be susceptible to developing pancreatic cancer as adults. Adult mice are excellent models in which to study pancreatic cancer for a number of reasons. We have a great deal of knowledge of the genetics of mice, and genetically altered mice carrying many of the gene mutations found in human pancreatic cancers already exist, meaning that we can combine these genetics quite easily. Further, the pancreatic tumours that develop in our mouse models go through the same stages of tumour progression that we see in patients, and spread to the same organs as in humans. Finally, pancreatic cancer is an incredibly complex disease that has a dense microenvironment, almost like a healing wound, which surrounds and supports the tumour cells and can account for up to 90% of the tumour volume in human pancreatic cancer. All the parts of this environment play an important role in disease progression and help tumours protect themselves from therapy. Importantly the pancreatic tumours in our mouse models also feature all of these components and are just as difficult to treat with drugs.





124. Parkinson’s disease: understanding changes in the brain and testing new treatments

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

No answer provided

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 is the aim of this project?

The aim of the project is to study the changes that occur in the brain of a rodent model of early stage Parkinson’s disease and to test possible treatments to prevent these changes and slow down the progression of the disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Little is known about what causes some of the early signs of Parkinson’s disease, like the loss of smell and memory loss. However, it is crucial to understand the causes of these signs to be able to develop better treatments. These symptoms can happen more than 10 years before a diagnosis is made based on the start of the typical tremor and slowness of



movements seen in Parkinson's disease. Detection of the early changes in the brain may improve diagnostics and new treatments for the disease.

What outputs do you think you will see at the end of this project?

Parkinson's disease, like other age-related neurodegenerative diseases, is increasing within the population as the average age increases. The number of people diagnosed in the UK is about 145,000 (around 1 adult in every 350). Current Parkinson's disease treatments are only targeting the symptoms but fail to stop the progression of the disease. In order to find new treatments, we need to determine the early cause of the disease. In this project, we aim to collect new information on the changes that occur in the brains of appropriate rodent models of Parkinson's disease and test new treatments that will rely on stopping disease progression or using the capacity of the brain for self-repair. All data will be presented at national and international conferences and published in scientific journals.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Scientists/doctors studying the origin of Parkinson's disease- The most effective clinical advance outside an outright cure for the disease may be an early diagnosis. Detection of early symptoms, like the loss of smell for example, in early-stage Parkinson's patients may greatly help such a diagnosis and allow treatments to prevent the death of nerve cells and stop the appearance of the more severe symptoms. To this end, appropriate rodent models that display symptoms and pathologies actually seen in patients will be a benefit to scientists and doctors who are trying to understand the origin of the disease.

Patients suffering from Parkinson's disease- The study of models of early stage Parkinson's disease that present symptoms similar to those displayed by Parkinson's disease patients may play a vital role in understanding the origin of the disease and determining promising treatments. Furthermore, it is vital to determine if some of the new drugs to be tested can also be effective in decreasing the symptoms observed in patients long before the typical shaking and slowed movements. The clear rescue effect of some new treatments in rodent models represents a very promising avenue for a new and long-overdue effective therapy for Parkinson's disease.

Scientists/doctors studying other diseases caused by death of brain cells- Patients suffering from these diseases- Information from this project could also prove very useful to scientists studying the causes and treatments of other serious disorders that affect brain function such as Alzheimer's disease or multiple sclerosis that share loss of smell as a common early symptom with Parkinson's disease.

In the lifetime of the project (5 years), we expect to build the knowledge base on the aetiology of the early stage of Parkinson's disease, to determine the effects of dopamine agonists and probiotics on non-motor symptoms observed in the early stage PD model and to compare these to those observed following EX-4 treatment. All data will be disseminated and presented in international conferences and published in peer-reviewed journals.

The EX-4 gene therapy, considered as drug development, is expected to have medium term benefits. Although the benefit of the drug itself has been previously shown, the project may lead to a novel method of administration that will carry less adverse effects than the drug treatments available for PD at present. This project, if successful, will see



our involvement in a future clinical trial as a medium term benefit. Long term benefit for this study will be the translation of this research into practice.

How will you maximise the outputs of your work?

All data will be presented in national and international conferences, discussed with experts in the Parkinson's disease field and published in scientific journals. Follow-up funding based on the data collected will be sought.

Species and numbers of animals expected to be used

- Mice: 300
- Rats: 470

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will be subjected to injections and surgical procedures via injection of a placebo solution, a toxin or a virus directly into the brain to induce the model of early stage Parkinson's disease (PD). Behavioural experiments will then be carried out at different relevant time points of the project to study both the effect of the injections and surgery (early stage PD model) and the effects of EX-4 that will be either injected or surgically administered as a gene therapy. The effect of conventional early stage PD treatments will also be tested and compared with that of EX-4.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

It is important to consider that our intention is to look at early stage Parkinson's disease. As such, the changes that are likely to be introduced will almost certainly be mild. To this end, we will be looking to use minimally-effective doses of toxins that give a cumulative but still subtle effect in the animals tested. Animals may suffer from weight loss for a few days after the surgery. We do not expect abnormal behaviours from rodents during the 4 week experiments. However, animals may start moving slowly and experiencing some motor disruptions in longer 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 species)?

This study will be of moderate severity due to the surgical procedures. We expect some variability in the speed of recovery between animals. However, based on past experience, we do not expect animals to show any adverse effects 3 days after surgery.

What will happen to the animals at the end of the study?



- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals will be used in this project to answer important scientific questions related to human health. The lack of a cure for Parkinson's disease or even the lack of treatments aiming to slow down the progression of the disease have meant that the study of animal models mimicking the early stage of the disease may bring new therapeutic targets to light and therefore new drug treatments for the condition.

What was your strategy for searching for non-animal alternatives?

Full or partial replacement would not be possible to mimic Parkinson's disease with a view to test new therapies.

Why were they not suitable?

The mammalian brain is almost certainly the most complex biological system that exists. Parkinson's disease is the result of disruption of several chemicals that have a unique but interlinked distribution in the brain. Replicating this in a dish would not be possible. Furthermore, the interaction between genetic mutations and toxins are also difficult to reproduce in non-animal alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals needed for this project was estimated using power calculations based on previous experiments carried out in our laboratory. Typically, each animal used will provide a large amount of data towards more than one objective.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Advice taken from local statisticians and the use of the results from preliminary experiments obtained on a previous licence, helped in the design of future experiments. The amount of data/information gathered per animal in one single experiment will be maximised in order to reduce the number of animals to be used in this project and answer all the questions set in each of the objectives. Negative results will also be reported to prevent unnecessary studies to be conducted by other laboratories.



What other measures apart from good experimental design will you use to minimise numbers?

Training of personnel will be compulsory for all the techniques used in this project to ensure good practice and reproducibility of the results. Tissues (i.e. brains from all experimental groups) will be shared, increasing the amount of data collected from each experiment. Pilot studies with a few animals will be carried out for each new drug to be tested to ensure that the best dose regimen is used and animal welfare is appropriately addressed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Our intention is to induce an early stage model of Parkinson's disease in both rats and mice to study the early causes of the disease in the brain and to test new or established early-stage treatments. These models will therefore not suffer from the more severe side effects associated with later stages of the disease as they will be culled either before the appearance of the motor symptoms or when these symptoms are just starting to show. We have extensive experience in the method used for the induction of the models and staff will follow appropriate guidelines to perform the surgeries on these animals. Daily monitoring after the surgery will be carried out to assess the behaviour of the animals and minimise pain, discomfort and stress during the experiments.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Most patients suffering from Parkinson's disease start developing symptoms at the age of 50 or over and it is rare that patients are diagnosed with the disease when they are under 40. We cannot therefore use animals at a more immature stage of life if we are to study the aetiology of the disease. The induction of an appropriate model of Parkinson's disease requires the use of adult rodents in which the loss of the nerve cells and appearance of relevant symptoms mimic what is seen in patients.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We consider the welfare of the animals of paramount importance. The post-operative surgical care will help prevent and minimise complications related to anaesthesia and surgical procedures. We do not expect complications following the procedures in this project. However, animals will be given recovery gel (DietGel recovery), a nutritionally fortified water that aids with post-surgical recovery, and the use of analgesics will be



considered pre-emptively and/or as needed after the surgery to minimise pain following close monitoring of the animals.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will ensure that experiments are conducted in the most refined way by following standard operating procedures from the Home Office, the LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery (E Lilley and M. Berdoy eds.) and the guidelines for the use of animals [Animal Behaviour (2018)].

How will you ensure you continue to use the most refined methods during the lifetime of this project?

My link to the NC3Rs through the newsletter and social media allows me to keep up-to-date with any advances in this domain. Any new development of new 3Rs technologies related to this project will be scrutinised and implemented if possible.

Explain the choice of species and the related life stages

Previous scientific studies have shown that it is possible to produce models of Parkinson's disease (PD) in rats and mice that display similar symptoms to those observed in patients suffering from the disease. Young adult rodents will be used for this study to avoid undesirable side effects of the injected toxins in younger animals. Rats are the most commonly used animal in PD research and are a reference point to help compare results with previously published data. We will also establish the early stage model in mice to allow comparison with available genetically-modified mouse models.

The duration of the experiments will depend on the different objectives of the project. The majority of the experiments will be terminated after 4 weeks. However, some will be continued until the rodents start experiencing some motor symptoms. A close monitoring will confirm the beginning of the symptoms and will dictate the end of the study.





125. Pathogenesis of Respiratory and Persistent Virus Infections

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Virology, Immunology, Pathogenesis, Vaccines, Anti-viral therapies

Animal types	Life stages
Mice	adult
Hamsters	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 is the aim of this project?

This programme of work aims to study the how the body responds to virus infections and use this information to investigate the potential new vaccinations and drug treatments. The viruses that we are studying are Influenza A virus (IAV), SARS-CoV2, human respiratory syncytial virus (HRSV) and gammaherpesviruses (γHV).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 viruses we are studying cause significant disease and death worldwide. SARS-CoV-2 is causing significant morbidity and mortality worldwide and may become a persistent



problem. Influenza causes seasonal outbreaks of disease and deaths in the elderly with occasional worldwide outbreaks that cause thousands of deaths each year. HRSV causes seasonal colds but is a major problem in newly born children, causes lung inflammation and can lead to death. Gammaherpesviruses cause cancers.

There are still large gaps in our knowledge of how these viruses cause disease and even where available, vaccines and antiviral drugs are not 100% effective due to virus evolution. This project will advance this knowledge and help us to design and test new drugs and vaccines. This will lead to reduced disease and deaths due to virus disease.

What outputs do you think you will see at the end of this project?

The viruses we are studying cause significant disease and death worldwide. SARS-CoV-2 causes significant morbidity and mortality worldwide. Influenza causes seasonal outbreaks of disease and deaths in the elderly with occasional worldwide outbreaks that cause thousands of deaths each year. HRSV causes seasonal colds but is a major problem in newly born children, causes lung inflammation and can lead to death. Gammaherpesviruses cause cancers.

There are still large gaps in our knowledge of how these viruses cause disease and even where available, vaccines and antiviral drugs are not 100% effective due to virus evolution. This project will advance this knowledge and help us to design and test new drugs and vaccines. The data from this programme will directly generate primary research publications from this work as well as presentation at scientific conferences.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short-term, it is mainly the scientific, medical and veterinary community working in basic research who will gain new knowledge.

During the life-time of the programme we aim to engage with clinicians and commercial entities to work towards translating knowledge obtained on anti-viral drugs and vaccines into the clinic. We also aim to use knowledge to inform policy and the public through the media.

In the long-term, our work might end up in the development of new therapies and vaccines in the clinic

How will you maximise the outputs of your work?

Most of this programme of work and the funding to perform it is already undertaken in collaboration with other scientists. We will present our findings as posters or oral presentations at international conferences, we will publish pre-print manuscripts on open-access servers like BioRxiv, which allows public scrutiny and feedback on our results by anybody interested in the work and we will publish all data, positive or negative in open-access formats to widen dissemination.

Species and numbers of animals expected to be used

- Mice: 4300
- Hamsters: 1000



- Rats: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mice, genetically modified mice and hamsters will be infected with viruses and these will cause disease in the lungs and sometimes cells of the immune system. We will also in some experiments test anti-viral compounds and potential vaccines. They will usually be maintained over a period of 2 weeks. The general condition of animals and their weight will be regularly monitored. Many of the animals will lose weight and then recover. About half of the mice will show no apparent sign of infection whereas the other half may show a mild flu-like illness.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The general condition of animals and their weight will be regularly monitored. Many of the animals will lose weight and then recover after about seven days. About 90% of animals may show a mild flu-like illness the rest will show no apparent sign of infection.

In a small number of mice we will alter the immune response. In these mice a more profound or rapid disease could develop after virus infection. In this case, the general condition of animals and their weight will be regularly checked. About 90% of animals may show a mild flu-like illness the rest will show no apparent sign of infection.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

About 90% of animals may show a mild flu-like illness which would be classified as moderate severity the rest will show no apparent sign of infection.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?



The proposed programme looks at how viruses cause disease in mammals. The interactions of multiple types of cells that form the lining of the lung, with cells of the immune system are needed to for our studies. These interactions are currently not possible in cell culture experiments in the laboratory. The lungs and immune systems of the animals we will be using are similar enough to recapitulate disease and it's mechanisms for us to study.

What was your strategy for searching for non-animal alternatives?

We already use cells in culture, 3D cultures that attempt to mirror airways of the lung and slices of lung to study aspects of how viruses cause disease in the lung.

Why were they not suitable?

Whilst we use cells cultured in the laboratory to study viruses and these experiments are informative, they are not currently able to mirror the complex environment found in the lungs and in particular how cells of the immune system interact with lung cells to defend against 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?

We have estimated these numbers of animals in two ways. First, based on previous experience from maintaining and investigating genetically modified mouse models that are similar to what is proposed in this project. The number of animals necessary has also been determined by statistical analysis, consulting with a professional statistician. This will ensure that we use the minimum number of animals necessary for our experiments to be statistically significant.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We will use a use a number of measures to reduce the number of animals used

First, we will use statistical and design software e.g. the NC3Rs Experimental Design Assistant (EDA) and power-calculation software to design animal experiments, maximise the data readout from a minimal n-number of animals and obtain estimates of how many mice might be needed for a specific experiment.

For experiments where we cannot use formal software in advance to estimate sample size, for example in novel experiments where we do not have an estimate of the expected standard deviation, we will utilise a block design or the similar 'variable-criteria sequential stopping rule' (SSR) and in consultation with a professional statistician

This will ensure that we use the minimum number of animals necessary for our experiments to be statistically significant.



What other measures apart from good experimental design will you use to minimise numbers?

We will employ careful long-term project management to ensure that only the required numbers of animals are obtained at the appropriate time for experiments to be performed.

Wherever possible, we will use and offer tissues that we do not need for our own research to other parties in order to share resources and maximise the usage of the animals for multiple aims.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will be using conventional mice, hamsters and genetically modified mouse strains that are deficient in components of the host response to virus infection. The structure and function of the lungs of mice and hamsters is similar to that of the human. They are therefore excellent animal models, which reproduce the complex human disease characteristics.

Data from our previous and future experiments will be used to determine the minimum doses of substances required to work but avoid side effects. The shortest possible time of treatment will also be used to obtain experimental data

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Other species such as zebra fish and drosophila (flies) are increasingly being used instead of mice. However, neither of these alternative models have a respiratory tract or lungs and thus are unsuitable for our studies.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We already through experience from previous and others' work that have enabled current 'best practice' protocols. Where new techniques are developed to refine procedures (as above) we will aim to adopt them to improve our methods.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

All workers involved will use a range of best-practice guidance



Protocols, training resources and guidelines on best practices in animal experiments are available through the NC3Rs website. We will also follow the NC3Rs ARRIVE guidelines, and make use of the NC3Rs Experimental Design Assistant for new experiments.

There are also a number of training and resources published by the RSPCA and available on their website that will be used to guide our practice.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We regularly attend workshops and seminars, including those run by the NC3Rs that keep us up to date on latest developments. We also receive NC3Rs newsletters and check the NC3Rs websites for novel developments. We will adopt novel techniques and equipment as appropriate during the project.

Explain the choice of species and the related life stages

The proposed programme looks at how viruses cause disease in mammals and how we can develop new interventions. We therefore need to be able to use an animal model system that enables us to perform these studies. The lungs and immune systems of the animals we will be using are similar enough to recapitulate disease and its mechanisms for us to study.





126. Pathophysiological Mechanisms of Cardiovascular Diseases: A Proteomics Approach

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Mice	adult, pregnant, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

Our aim is to further our understanding of the processes that underlie cardiovascular diseases. We do this by using advanced techniques for protein analysis (proteomics). Proteomics allows us to understand the function of proteins that are produced by the body, and are the main building blocks of our blood vessels. In particular, we are interested in proteins constituting the structural scaffold of our vessels.

Starting from observations in patients, we will take advantage of genetically modified mice. These genetic modifications affect the building blocks that constitute the scaffold of our blood vessels, and the way these building blocks get modified over time and in disease. By doing this, we can investigate molecular targets towards which new potential drugs should be directed. These modifications do not only affect the structure of the vessels, but also the signals that are presented to the cells that populate the blood vessels (and that in turn, produce the building blocks), allowing us to expand the potential repertory of drugs and therapies aimed to prevent the changes in our vessels that lead to cardiovascular disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these



could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Ischemic heart disease and stroke are the two leading causes of death worldwide. Both conditions are mainly caused by atherosclerosis, a disease in which the inside of an artery narrows due to the build-up of plaques. The plaques are predominantly composed of fat and can rupture leading to the formation of a thrombus. Narrowing of the artery and subsequent thrombus formation limits the flow of oxygen-rich blood to parts of the body.

Besides acute mortality following these ischemic events, survival often goes hand in hand with severely diminished quality of life. While ischemic heart disease can lead to heart failure, strokes can cause lifelong morbidity. This research programme will provide basic knowledge useful for both scientific research and clinical practice. We aim to achieve this by discovering new drug targets or better methods of disease diagnosis that will benefit patients both in the UK and worldwide.

What outputs do you think you will see at the end of this project?

The proposed project will advance our knowledge on the remodelling process taking place in our vessels during cardiovascular disease. Other processes such as inflammation are also associated with cardiovascular disease and will also be studied to better understand the link between inflammation and remodelling of the vessels. Together, our research will potentially lead to a change of the clinical practice in the future in the form of improved diagnosis and novel therapies that will positively impact on the survival and the quality of life of patients. As per our previous work, all relevant data emerging from this project will be published at the highest possible level in the most appropriate scientific journals.

This will ensure not only the sustainability of our line of research and success in future funding, but also a maximal outreach for our research, so it is useful for others and paves the ground for new research, ultimately leading to a change of the clinical practice in the future.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The primary aim of our research is to decipher basic processes occurring during the remodelling of our vessels during cardiovascular disease. The processes by which the scaffold of our vessels gets modified during cardiovascular disease have not been fully studied. In the short term (i.e. <5 years), our research aims for the development of strategies that control remodelling and degradation of the scaffold of our vessels during cardiovascular disease. We will only use mice under this license. At a later stage, models, i.e. involving the use of gene therapy, will need to be expanded to large animals (i.e. 3-5 years) before research can be translated into patients. These studies will be performed by collaborators at other centres under other licenses. Ultimately, we hope that our research will be applicable to patients suffering from cardiovascular disease (i.e. up to 10-20 years), possibly in the form of better diagnostics or combined therapies that improve the efficacy of current therapeutic approaches, and that will have a positive impact in the quality of life and outcomes of patients.

How will you maximise the outputs of your work?



Our protocols have been adapted to our research questions based on previous experience from other research groups in our Centre. This is a strategy that we will continue to use in the future.

Hearts obtained from previous experiments focussing on vessels only have been used to generate preliminary data that secured a fellowship. This demonstrates the level of synergy that can be achieved in REDACTED, and our commitment to maximize the outputs obtained from our research whilst minimising the use of animals.

Our group has an extensive track record of collaborative research, demonstrating that data from our experiments can be complemented by research in other internationally recognised institutions in order to maximize the impact of our outputs.

As a general policy in our group, we publish our results and disseminate them at scientific conferences, including negative findings or unsuccessful approaches, so our research is helpful for others, and we ensure that we do not duplicate unnecessarily the research already done by others.

Whenever relevant, we do not only publish clinically relevant work, but also technical papers that make our protocols available for other groups.

The data are deposited in public data repositories. Together, these strategies ensure that our research contributes to maximal dissemination of knowledge.

We will engage with clinical partners to seek for feedback, ensure that our research is applicable to human disease, and that it covers actual clinical needs, so it can ultimately benefit patients and be implemented into the clinical practice in the future. When appropriate, we will get involved in the development of any potential clinical therapy emerging from the data obtained from (or part of) our research.

Species and numbers of animals expected to be used

Mice: 6,150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, mice under our license will be bred and maintained in order to provide a stock for our experiments. In most cases, animals will be kept for about a maximum of 20 weeks. This is sufficient time to perform most of the procedures described in our license. An exception to this will be animals used to investigate ageing, and animals used as breeders. The former will be kept for a maximum of 24 months, while the latter will be kept for a maximum of 12 months (males) or about 6 months (females).

Animals undergoing experimental procedures will typically enter experiments between the 6th and the 10th week of age and are generally culled before the 20th week of age, once



the experimental purpose has been achieved, avoiding any unnecessary suffering. Again, an exception to this is represented by animals used to study ageing (i.e. about 6% of the mice included in this license), on which similar experiments will be performed on a later time point in life.

We anticipate two standard routes our animals will typically follow:

The animal will first undergo an evaluation using echocardiography under anaesthesia, after which a surgery will be performed to induce the relevant vascular disease. After that, cardiovascular functions are monitored using echocardiography (once or twice) until the experiment is finished, usually within the first month after surgery, after which the animal is humanely culled.

The animal will first undergo an evaluation using echocardiography under anaesthesia, after which the animal will be, for example, fed a cholesterol-enriched diet to induce vascular disease. Animals are then maintained on this diet for about 12 weeks, after which a final monitoring is done using echocardiography, before the animal is humanely culled.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Most animals in our protocols are not expected to experience an impact to their wellbeing beyond that required for the experimental purpose. However, a risk of observing side effects is associated to each experiment, as described in our protocols. Any potential side effects will be closely monitored, and remedies provided to ensure that animals do not experience unnecessary or long-lasting suffering.

Typical examples of side-effects are as follows:

For experiments involving surgery: Complications can include bleeding, wound infection or breakdown of wounds.

For experiments involving diet manipulation: Complications can include weight loss, or excessive weight gain.

For experiments involving ageing of mice: The mice will experience symptoms of senescence like skin conditions (e.g. dermatitis and eczema), reduced activity levels, and weight loss. Other age-related side-effects may include tumours, heart failure or the presence of seizures, but these are only anticipated to affect a very small proportion of all aged animals.

For genetically modified animals: We will be using a mouse model of obesity. These mice eat more than normal mice, become obese, and therefore they may display conditions commonly associated to obesity. These include impaired mobility, and occasionally the development of wounds that take longer than normal to heal. Other animals with genetic modifications are not expected to show side effects different to those appearing in normal mice.

Expected severity categories and the proportion of animals in each category, per species.



What are the expected severities and the proportion of animals in each category (per species)?

Examples and definitions of severity levels are provided at the end of this section. We will only use mice in our protocols. Estimates of expected severity levels under this license are as follows:

We will use an estimated total of 6,150 mice.

These animals will be breed and/or maintained at our facilities. The vast majority of these animals (6,000, i.e. 97.5%) will experience only mild suffering related to breeding and maintenance. Only 150 (2.5%) animals will suffer a moderate level of suffering related to breeding and maintenance.

Of animals entering protocols other than breeding and maintenance (4,350), 75% will experience a mild severity level, while 25% will experience a moderate severity level.

Mild severity is defined as that in which animals are likely to experience short-term mild pain, suffering or distress, or not significant impairment of the well-being or general condition of the animals. An equivalent example of this level of severity is an echocardiography or the administration of an anaesthetic.

A moderate severity level is defined as that on which the animals are likely to experience short-term moderate pain, suffering or distress, or long-lasting mild pain, suffering or distress as well as any procedure causing moderate impairment of the well-being or general condition of the animals. An equivalent example of this level of severity is a surgery under general anaesthesia and appropriate analgesia or frequent withdrawal of blood samples.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models are essential. Whenever possible, strategies not involving the direct use of animals have been implemented (tissue engineering, cell cultures). Unfortunately, the animal models proposed for this project cannot be replaced by other methods, since the complex process of atherosclerotic lesion formation and blood vessel remodelling cannot be accurately reproduced, and there is no suitable alternative to animal models for studying this complex and chronically developing condition.

What was your strategy for searching for non-animal alternatives?

Alternatives considered for our research include the development of tissue engineered scaffolds (e.g. artificial tissues) for experiments looking into the effect of stretch on blood vessels (similar to what happens during hypertension). For experiments looking into processes at the molecular level, we use commercially-available cell lines when possible,



or cells obtained from relevant mice. When feasible, we obtain clinical samples and data rather than using animals. We have collaborators and staff in our group dedicated to the development of biostatistical and computational models. All these alternatives will be used whenever feasible.

Why were they not suitable?

Certain processes that will be studied in this project using animals cannot be studied in cell culture studies nor can they be mimicked by computational modelling due to the numerous uncertainties/unknowns regarding these complicated diseases. The scaffold of our vessels is exposed to continuous forces, e.g. by high blood pressure, and this scaffold is formed over long time periods. These conditions can only be produced in vivo (i.e. in a living animal and not in cells). We will complement our studies in animals with in vitro studies (i.e. in a test tube, culture dish or elsewhere outside a living animal) to obtain greater detail. However, such studies in isolated cells/organs cannot model chronic changes and interactions among multiple body systems 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 to be used in the present project has been carefully estimated based on the funded research projects currently active in our lab and a reasonable estimation of future projects to be obtained during the duration of this project.

All our experiments are performed after careful statistical estimations based on preliminary experiments or relevant literature. All our current grants involving animal work have been only accepted after providing appropriate calculations regarding animal numbers, ensuring that funding is only available for the required animals. Importantly, when estimating total numbers, we have taken into account the fact that some animals might serve multiple purposes (for example, tissues used for different objectives). The majority of measurements we aim to obtain are suitable for statistical analysis. Dropout for the different models has also been taken into account in order to estimate final numbers.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We have implemented methods like echo imaging that allow non-invasive serial assessment during the experiment in the same animal, which significantly reduces the numbers required, as otherwise animals would have to be killed at each relevant time point to collect data. Serial assessments also reduce experimental variability by allowing comparisons at different time points in the same animal, thereby providing more statistically robust data. At the end of the experimental period, animals are humanely killed. Our protocols ensure that once samples are taken, the maximum possible data can be obtained from each animal. This also significantly reduces the total numbers of animals required.



Experimental study plans will be written for each experiment as part of good laboratory practice, to include: objective(s); description of the experiment including experimental treatments; the number of groups and number of animals/group; data to be obtained; and an outline of the planned method of analysis of the results. For genetically modified animals, where suitable mice already exist, they will be obtained from the relevant supplier. In cases where we generate the required genetically modified mice ourselves, we will measure production and breeding performance and ensure that the minimum numbers of animals required are bred.

What other measures apart from good experimental design will you use to minimise numbers?

All experiments proposed for this project will be designed based on pilot experiments that provide important data to help us to estimate the number of animals required on each individual experiment. We will keep records of breeding efficiency and survival, to help us to estimate the number of animals produced on each breeding cycle. All female breeders will be identified and tracked for the number of pregnancies and age, so only efficient breeders are used. When feasible, we will ensure that mixed animals will not be bred. This occurs when the parent animals have different genetic backgrounds; this will be avoided whenever possible. Whenever possible, tissues from the same animals will be used for multiple purposes and can be shared with other groups from which we might also obtain tissues (i.e. hearts from the same mice can be used for projects by other lab members), overall reducing the number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Only mice will be used. Mice have a well-defined genetic map and are sufficiently similar to humans to obtain clinically relevant conclusions. Mice require small quantities of reagents yet there is a wide availability of specific reagents that can be used for this species. Examples of these are antibodies and inhibitors that have been developed for mice, but would not be available for research in other models. These reagents are more specific and can be used in smaller doses, considerably reducing side effects and pain caused to the animals.

Atherosclerosis-prone mice (i.e. mice that are prone to present fatty deposits in their arteries leading to hardening of these blood vessels) develop mild disease even when on normal diet. When on Western diet, these animals require shorter times than control mice develop vascular disease. Therefore, our protocol ensures that mice are on protocols for minimal time periods. The scaffold of our vessels is critical for the binding of lipids to the vessel wall, and for the structure and stability of the vessel. Genetic modification of specific components of the scaffold of blood vessels allows us to study the effects on the structure and remodelling during the development of cardiovascular disease.



This project intends to use mainly mice which are prone to develop atherosclerosis or have defects on the scaffold of their blood vessel. The vast majority of mentioned genetically altered mice develop and grow normally and have normal fertility.

All methods used for the present project use the least invasive and least painful techniques of those available. Our group has adopted techniques that reduce operation time and chance of bleeding and increase success rate. These surgical techniques include the implantation of minipumps (it avoids repeated animal dosing by injection), telemetry probes are the gold standard in mice to measure blood pressure, and a simplified model of vessel grafting. These models have been reproduced by other laboratories and are well described in the literature.

Whenever possible, we choose to induce disease by methods not involving vascular surgery, as in the case of inducing high blood pressure. Several of the methods rely on the administration of agents with the diet or with drinking water (e.g. agents that cause high blood lipid levels and high blood pressure) avoiding the use of injections and reducing considerably the stress caused to the animals.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Our main goal is to study disease progression. Therefore, animals terminally anaesthetised are not appropriate. Species that are less sentient do not share sufficient common characteristics with humans, nor are they sufficiently characterised to be reliably used as models of human disease. Animals are used at the earliest possible life stage, but the composition of the scaffold of blood vessels changes and builds up over life, and we can only use mice in which a fully mature blood vessels have developed (i.e. after 8 weeks of age).

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

All surgical procedures under all Protocols will be conducted under sterile conditions to avoid infections, with appropriate pain relief, the highest levels of post-operative care and appropriate veterinary consultation. As the major component of mortality or expected side-effects is during surgery and in the first 24 hours after surgery, animals will be closely monitored at frequent intervals during this period. Animals will be reviewed at the end of the working day on the day of surgery and any considered likely to die overnight will be humanely killed. Careful attention will be paid to heating, pain relief, body weight, surgical wound-sites, hydration, and signs of pain or distress.

We periodically revise all medications administered to our mice; e.g. we have recently changed from an injectable to an oral pain medication. We periodically re-assess all methods with the help of other members of our department.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

LASA guidelines

Guidelines and training materials provided by NC3R



How will you ensure you continue to use the most refined methods during the lifetime of this project?

Websites e.g. <https://www.nc3rs.org.uk>, <https://www.lasa.co.uk>

Webinars e.g. hosted by Charles River

Advice of NVS, NACWO, NTCO and NIO

Attendance of scientific conferences

Publications in cardiovascular journals

Explain the choice of species and the related life stages

Only mice will be used. Mice have a well-defined genetic map and are sufficiently similar to humans to obtain clinically relevant conclusions. Mice require small quantities of reagents yet there is a wide availability of specific reagents that can be used for this species. Examples of these are antibodies and inhibitors that have been developed for mice, but would not be available for research in other models. These reagents are more specific and can be used in smaller doses, considerably reducing side effects and pain caused to the animals.

Atherosclerosis-prone mice (i.e. mice that are prone to present fatty deposits in their arteries leading to hardening of these blood vessels) develop mild disease even when on normal diet. When on Western diet, these animals require shorter times than control mice develop vascular disease. Therefore, mice are on protocols for minimal time periods. The scaffold of our vessels is critical for the binding of lipids to the vessel wall, and for the structure and stability of the vessel. Genetic modification of specific components of the scaffold of blood vessels allows us to study the effects on the structure and remodelling during the development of cardiovascular disease.

The Figure below compares the life phases and maturational rates of mice and humans. Mature adult mice range in age from 3 - 6 months, which is the life phase equivalent for human ranges from 20-30 years. For experiments not involving ageing, we generally choose to use animals up to the 6th month of life. While these are young adults, they are mature animals, and this prevents the introduction of co-founding factors related to age that are often not relevant for our projects and might mask our results. Animals of older ages also tend to present with higher variability (e.g. broader weight ranges), and often require the use of larger amounts of reagents. Ageing will be a specific matter of study in some of our projects. For these projects, mice will be kept up to the age of 24 months. Noteworthy, the age range 18-24 months in mice is equivalent to the 6th and 7th decades of life in humans, at which most cardiovascular events occur. This age range is therefore highly relevant for studies looking at the changes in blood vessels occurring upon ageing.

<https://www.jax.org/news-and-insights/jax-blog/2017/november/when-are-mice-considered-old>





127. Physiology of the extracellular space of the brain and its role in neurodegeneration

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Rats	neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

This project aims to understand:

how protein aggregates are removed from the space in between brain cells during wake and sleep

how individual protein aggregates may interact with receptors on neurones inducing toxic effects

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neurodegenerative diseases are a heavy burden for individuals and society as a whole. By 2016, the global number of people with dementia was estimated at 43.8 millions (Lancet Neurology 2019; 18: 88– 106), and this number is clearly on the rise. It is estimated that there are over 850,000 people living with dementia in the UK and that this costs the UK £26 billion a year. My project will address key aspects of dementia in a highly



interdisciplinary way, aiming to clarify how the (suspected) main toxic agents which induce neurodegeneration are cleared out of the brain.

What outputs do you think you will see at the end of this project?

The main output will be in form of scientific publications. We will present this work in national and international meetings as well.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

To animals:

The project aims to understand how cerebrospinal fluid (or interstitial fluid, i.e. the liquid in between cells in the brain) flows inside the brain and how this affects the clearance of toxic protein aggregates (or protein particles) from the extracellular space of the brain. This has been a matter of debate for the past 50 years, and it is currently becoming an important topic in the field of neurodegeneration. The clearance of protein aggregates is an issue that affects animals in general, not only humans. Mounting evidence suggests that there are a variety of species that suffer different forms of amyloid and/or tau pathologies, including domestic dogs, domestic cats, a variety of non-human primates, REDACTED, black bears etc. Furthermore, we'll study how protein aggregates circulate this convoluted space and how does the complexity of the extracellular space of the brain affect the clearance of these aggregates and the likelihood of interactions between aggregates and receptors in neurons. Understanding the role of sleep in clearing toxic metabolites from the brain might also provide valuable insights into welfare of animals whose natural sleep patterns are disturbed by artificial light.

To humans:

We will provide novel experimental evidence in order to clarify the current controversies on the mechanisms by which the brain clears protein aggregates present in the spaces between cells. This is a fundamental issue in the field of neurodegeneration, and in particular in the field of Alzheimer's disease research. The results will also help to understand the risks that modern society faces with bad quality sleep due to the abundance of electronic devices that have been reported to interfere with good sleep habits in a large fraction of society, particularly among children. We will also study what is the direct toxic mechanism of protein aggregates when they interact with receptors in neurones, in particular the interactions of alpha-synuclein aggregates (relevant to Parkinson's disease) and amyloid- beta aggregates (relevant to Alzheimers disease) with a selected subset of their putative receptors. These interactions have been reported to impair memory formation and learning among other deleterious effects.

To scientific knowledge:

This project addresses both physiological and pathological issues of the brain that could not be studied until now due to the lack of suitable biophysical techniques. We aim to provide a better understanding of the (almost unexplored) physiological characteristics of 20% of the mammalian brain, and how is this regulated in wake and sleep. The consequences of this study have the potential of opening new avenues in Alzheimer's disease research.



How will you maximise the outputs of your work?

Our work is highly collaborative. We will collaborate and share results with top leaders in the field, including my established collaborators REDACTED, USA and the UK. Funded by the European Commission, I have opted into a Pilot project to share data online in a public way, so many other research groups can also benefit from this study.

Species and numbers of animals expected to be used

Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The project requires the injection of tracers (small fluorescent particles, fluorescent dyes or proteins) to follow the flow of cerebrospinal fluid in the brain, as well as tracers to describe the behaviour of relevant actors in the mechanism under study. Once the tracers are in the brain, we need to study the trafficking of the tracers, the state of the extracellular space and the dynamic behaviour of related molecules. For this purpose we need to analyse the brain and cerebrospinal fluid using a variety of microscopy techniques, which require the preparation of brain and cerebrospinal fluid samples as well as the direct observation of the brain in a living (anaesthetised) animal through a cranial window.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

All procedures in the project will be done under anaesthesia, and in most cases this anaesthesia will be terminal. In one out of the three procedures needed, the animal will be injected with the necessary substances in the brain and then it will be allowed to recover. In this case, the animal will be given the necessary analgesic doses and will be closely monitored. As all the substances that we need to inject are biocompatible (within the timeframe of the experiments) we do not expect any abnormal 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 species)?

Two of the protocols are done under terminal anaesthesia, so animals will not recover. One protocol (injection of substances to the brain under anaesthesia) will allow the animal to recover, so we classify the severity as moderate.

What will happen to the animals at the end of the study?

- Killed



Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To study the organisation and function of the space in between cells of the brain we cannot use cultured cells, as the native architecture of the brain is needed. We do not have an in vitro model to replicate the complexity of the brain's extracellular space. Furthermore, in order to address the differences between the physiology of the extracellular space of the brain during wake and sleep we need to work with living animals. Nonetheless, whenever possible, we will replace the experiments in vivo by using primary or organotypic cultures.

What was your strategy for searching for non-animal alternatives?

I considered using cultured neurons, mixed cell cultures and organoids.

Why were they not suitable?

Although cultured neurons (or even cultured brain slices) are useful systems to investigate some neural mechanisms, they unequivocally differ in many aspects from cell networks in intact brain preparations (acute brain slices). For instance, the architecture of the cellular assemblies is strongly altered, causing changes in the extracellular environment and intercellular communication.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 proposed project has been estimated from experiments I conducted during 4 years of work in REDACTED. There, REDACTED the tools to do single-nanoparticle tracking in brain slices and settled the experimental basis for the project I propose to do. The experiments proposed here have not been done before (the experimental techniques needed are not available elsewhere), so we are not able to predict a precise number of animals through statistical calculations. Nevertheless, based on the experiments I have done, I am confident that I can predict an upper limit to the number of animals that we will use.

To reduce the number of animals in the study we will work with cultured brain slices to obtain information about the organisation of the extracellular space of the brain, the abundance of cul-de-sac structures in the brain, the variation in complexity across different parts of the brain, and the regulation of the extracellular space volume. In vivo experiments will be necessary to take into account the effect of cerebrospinal fluid flow and blood



pressure in extracellular trafficking, as well as the dynamical changes of the volume of the extracellular space of the brain between wake and sleep. In the case of the interactions between receptors and protein aggregates, every receptor under study will be first characterised in cell lines, subsequently in primary cell cultures and brain slices, and only once these experiments are done the overall model will be validated in vivo.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

To estimate the numbers of animals needed we have extrapolated from similar studies previously carried out and made some educated guesses.

What other measures apart from good experimental design will you use to minimise numbers?

I will do computer modelling to explore the behaviour of the system and have more accurate experimental result. As I have done in the past, simulations of receptor mobility can help us to predict behaviour and narrow down the experimental parameters to be explored.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The model organisms to be used will be Sprague-Dawley rats, since they have a suitably advanced nervous system for investigating the mechanisms we want to address. This is the only species in which the extracellular space of the brain has been characterised in acute brain slices at the scale in which events addressed in this proposal are regulated (i.e. with a spatial resolution below 100nm).

Furthermore, it is the only species in which the mobility of receptors has been characterised in brain slices at the single-molecule level. The proposed project will remain in the same animal model in order to maximise impact in the field by being able to compare results with the work of other research groups. Furthermore, this choice will decrease risk, workload and number of animals needed.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Two out of the three protocols needed are done under terminal anaesthesia. It is not possible to do the three protocols in this condition because we need time for the injected substances to diffusion along the brain. We cannot use more immature animals because the barriers in the brain are not fully formed and the physiological effects under study will not be fully functional.



What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

To minimise the suffering, experiments have been designed in such a way that all stereotaxic surgeries are done under general anaesthesia. Out of these surgeries, 75% will be terminal and the other 25% will allow the animals to recover while they are treated with analgesics. All experiments encompassing cranial windows will be under terminal anaesthesia. In all cases we will use appropriate peri operative care measures as advised by the NVS and surgeries will be carried out under aseptic conditions.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The studies will be done in different levels of complexity: cerebrospinal fluid analysis, experiments in cultured cells, in cultured slices, in acute slices, and finally in vivo. These systems are routinely used worldwide in neuroscience laboratories, with well-established protocols that guarantee reproducibility of preparations and are robust enough to be compared across different laboratories. Protocols for dissociated primary cell cultures are commonly used for imaging purposes. Protocols for brain slices are commonly used when connectivity matters, as it is the case in electrophysiology studies and imaging studies that lack meaning in dissociated cells. The protocols for in vivo imaging experiments are equivalent to the well established in vivo 2-photon microscopy experiments performed in many laboratories worldwide. Additionally, we will use the ARRIVE guidelines to design, perform and report our in vivo experiments.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Discussing possible improvements to experimental conditions with technical staff and obtaining new ideas for improvement in forums such as LASA and NC3R.

Explain the choice of species and the related life stages

The main goal of this project is to study the mechanisms by which the brain clears out small protein aggregates from the brain, in particular the aggregates that are located outside cells. Among these protein aggregates we will focus on the ones that are thought to be the cause of neurodegenerative diseases such as Alzheimer's or Parkinson's. In order to study how the brain naturally clears out these toxic agents, we need a model of study that has the physiological mechanisms relevant to this process. This can only be achieved using living animals, at ages in which the barriers between compartments in the brain are already formed and fully functional. The model organisms to be used will be Sprague- Dawley rats, since they have a suitably advanced nervous system for investigating the mechanisms we want to address. This is the only animal in which we have a detailed characterisation of the space in between cells, which is very complex and dynamic. The proposed project will remain in the same animal model in order to maximise impact in the field by being able to compare results with what has been published about the extracellular space in the living brain. This choice will decrease the risk of the project.





128. Polyclonal Antibody and Normal Serum Production

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

No answer provided

Animal types	Life stages
Sheep	adult
Goats	adult
Alpaca	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The Aim of this Licence is to provide a high quality antibody and normal serum production service to the Scientific Community throughout the World.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Antibodies and normal serum is used by Medical Diagnostic Companies worldwide for the manufacture of Test Kits for the detection of disease in both The Human and Veterinary markets.

Diagnostic kits and reagents are used by Blood Banks and Hospitals throughout the world for the detection of common bacterial and viral diseases that include but are not limited to Hepatitis, MRSA, Salmonella, Yellow Fever, Shigella and Streptococcus infections.



It is a legal requirement to test donated blood for a range of diseases.

Antibodies are also used to better understand disease and dysfunction to further knowledge in a wide range of health conditions in man.

What outputs do you think you will see at the end of this project?

The Project will deliver outputs in the form of high quality antibodies throughout the life of the Licence. These outputs will be used immediately in products many of which are gold standard tests used in the ongoing testing in Hospitals and Blood banks on a daily basis throughout the World.

Where the project is delivering antibodies for research or vaccine development the outputs may take a longer to deliver benefit, these outputs may take the form of publications and new information.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The Business manufacture high quality IgG antibodies for use in diagnostic test systems that are used for the identification of infective agents and physiological disorders.

Prompt and effective treatment is required in some diseases where a severe infection or fatal outcome is likely.

E.g. Bacterial Meningitis infection in children, where rapid detection is required so that appropriate treatment can be administered without delay.

Early identification of an infectious agent can be of considerable value in providing the patient with appropriate antimicrobial treatment and minimising the risk of spread of infection to susceptible individuals.

How will you maximise the outputs of your work?

The Business will work closely with clients to record data on antibody response, initially to ensure that minimum severity is used to achieve objectives but secondly to maximise outputs.

Objectives can be measured as titre level of response and volume of antisera generated.

This plan of work will be progressed by strong data gathering and regular discussions with clients, ERP meetings will used to drive the initiatives.

Species and numbers of animals expected to be used

- Sheep: 950
- Goats: 450
- Camelids: No answer provided

Predicted harms



Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will be injected with a set of antigens a minimum of 14 days apart to evoke an immune response.

In the short term 6-9 injections will be carried out over 3-6 months.

Adjuvants will be used in conjunction with the antigens to improve response. Antibodies will be produced in the animals blood system, these will be harvested by the removal of blood from the animal on a monthly basis or by terminal harvest.

Depending on the type of work animals will either be humanly ended where all blood will be harvested.

Where the work is on a longer term basis animals will be kept as donors to supply up to 10 blood samples per year.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Animals may experience mild reactions at injection sites, these are rare, during the blood harvesting phase animals may suffer anemia.

There are set procedures to monitor all potential side affects which include monitoring the blood of each individual to ensure that animals are fit and healthy, individuals may be rested and supplemented with vitamins to ensure health is maintained. Each animal is monitored for early signs of reaction to the antigen.

Animals kept for long term supply of blood will be given two rest periods per year.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

All animals are classified as Mild severity

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.



Why do you need to use animals to achieve the aim of your project?

Sheep, Goats & Camelids have been historically used for antibody production, they were originally chosen for their ease of use (blood sampling & antigen dosing), plentiful supply and ability to produce high quality antibodies.

Currently there are no methods available for the production of specific polyclonal antibodies using non animal alternatives.

What was your strategy for searching for non-animal alternatives?

Production of polyclonal antibodies is not achievable without a living mammalian host.

Why were they not suitable?

No answer provided

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of animals stated are based on historical use.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Reductions of 30% have been achieved in the last 12 months by maximising techniques for the recovery of blood and processing in the Laboratory, this initiative will be continued.

What other measures apart from good experimental design will you use to minimise numbers?

Purpose bred animals that produce larger volumes of serum due to their size and weight (purpose bred strains) will be used where possible, these animals will be bred on site.

A further 25% reduction of animal use has been achieved in the last 2 years with this approach.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.



Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Sheep and Goats have been historically used for antibody production, they were originally chosen for their ease of use (blood sampling & antigen dosing), plentiful supply and ability to produce high quality antibodies.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Adult, mammalian animals are required for the the generation of antibodies used in medical diagnosis of disease in Humans.

Sheep, Goats and Camelids are well suited for this role.

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Refinement is achieved in many ways including; use of disease free stock, an ongoing training / coaching system of staff to ensure good welfare, environmental enrichment, objective health monitoring, and regular assessment of individual staff and their procedural techniques.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

NC3R's

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Regular meeting attendances and effective ERP meetings.

Explain the choice of species and the related life stages

Sheep and Goats are commonly used for the production of polyclonal antisera. Alpacas and Llamas are used to provide a unique type of antibody.

Adult animals are used as they have well developed immune systems and peak weights which ensures good antibody production and larger volumes of harvested blood.





129. Preclinical safety, efficacy, and pharmacokinetics of antimicrobial peptides and antibiotics for corneal infection

Project duration

2 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- 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

No answer provided

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 is the aim of this project?

The overarching aim is to assess the safety, efficacy, and key biological measurements (pharmacokinetics parameters) of our newly identified antimicrobial peptides alone and/or in combination with antibiotics in mouse model of wound healing and corneal infection

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Clinical need: Cornea is a transparent window in the front of the eye. Corneal diseases are the 5th leading cause of blindness globally[1]. In addition it accounts for 2 million new cases of single-eye blindness every year[1]. Corneal infection represents a major cause



for corneal blindness worldwide, with contact lens wear and trauma being the main risk factors[2]. *Pseudomonas aeruginosa* (PA), a bug, is the commonest cause of corneal infection with worst outcomes of disease. Lack of effective therapy and constant rise of multi-drug resistant (MDR) bugs have increased the prevalence of blindness[2]. One of the strategies of the World Health Organization (WHO) is to improve the effectiveness of existing antimicrobial agents and/or development of alternative drugs[3]. Vancomycin (VCN) is a last resort antibiotic used for treatment of difficult to treat infections caused by a bug known as methicillin-resistant *Staphylococcus aureus* (MRSA) [4]. However, it does not cross through the surface (outer membrane) of another bug (Gram-negative bacteria including PA) and therefore less preferred for clinical treatment[5].

Solution: Antimicrobial peptides are naturally occurring host defense molecules with killing activity against a wide range of disease causing bugs[6]. FK13 and FK16, the small size peptides, have previously shown greater killing effectiveness against a variety of bugs such as bacteria and fungi[7]. We have successfully showed that FK16 *has* specialised abilities (creates hole on the surface of bacteria), which helped improving the bug-killing capability of antibiotic [8].

However, thus far, it is unknown whether the peptide/antibiotic combination therapy is ready for the management of corneal infections. It is anticipated that the proposed animal studies will confirm the safety and efficacy of this new therapy and provide a platform to undertake more advanced studies and early clinical trial for the treatment of corneal infections in humans.

REDACTED

What outputs do you think you will see at the end of this project?

The key output will be the preclinical safety, efficacy, and key biological data (pharmacokinetic parameters) for the proposed peptide/antibiotic combination drug. These outcomes will be protected as intellectual property and lay a strong platform to test our new drug therapy in human-based clinical trials.

The results of this study will also form the basis for high-quality publications and impact the research excellence framework (REF) outputs of our REDACTED.

Pseudomonas aeruginosa is an opportunistic bug (a bacteria) that also affects other bodily organs such as kidney and lungs. Thus, outputs of this proposed study will be utilised to gain translational REDACTED to test this new combination therapy in the animal models of renal and lung infections.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Who will benefit?

Patients and the health services

Industries and policymakers

How it will benefit?



Benefits to patients and the health services: Eye infection caused by bacteria is one of the leading causes of single-eye blindness and other life-threatening bodily infections. The proposed new drug treatment could considerably improve patient's quality-of-life, ultimately reducing overall burden on the UK's national health services and global healthcare systems.

Benefits to industry and the policy makers: The discovery arising from this study will be commercially protected as an intellectual property (also known as a 'patent'). With an added advantage to utilise this drug therapy for other bodily infections, pharmaceutical manufacturing firms will be benefited. This will improve their economic output and eventually their budget for translational research. The research outcomes will impact the policy makers/regulatory bodies, promoting fast-track approvals for patient- based studies and implementation of our drug therapy for the treatment of infections.

Timescales of impact:

With the successful outcomes from this research, we have plans to initiate advanced animal and early clinical safety trials (Phase I) within 12-18 months. These studies will be complete in 2-3 years. Next, a larger phase II/III trials will be conducted to assess the efficacy of therapy in patients with corneal infection. The culmination of phase II/III trial studies (up to 3-5 years) will provide necessary clinical data to seek for the fast-track MHRA (Medicines and Healthcare products Regulatory Agency) and FDA (Food and Drug Administration) regulatory approvals for using our new therapy combination for treatment of infection.

How will you maximise the outputs of your work?

This study is now funded through the institutional support by the UK Medical Research Council under the Confidence in Concept scheme. It is anticipated that the outputs will include meaningful preclinical efficacy and safety data, which will form the basis for advanced studies through translational funding from the MRC Developmental Pathway Funding Scheme (DPFS), National Institute of Health Research (NIHR) i4i scheme or UKRI (Innovate UK scheme) within 18-24 months of completion of this project.

Dissemination Plans:

Corneal infection is an avoidable blindness condition. Within 6-12 months of completion of study, my group will engage in educational activities targeted at young people and adults to promote awareness of hygiene and proper use of contact-lens and their implications to blindness. Additionally, we will also involve societies/charities such as Fight for Sight (FFS), National Eye Research Centre (NERC) and Sight Savers International (SSI) and Royal National Institute of Blind People (RNIB) to disseminate research findings, receive lay-man reviews and building capacity for future research. To disseminate our findings, we will ensure timely publication of our research in high impact peer review journals and through conference attendance/presentation (national and international). Providing there is no copyright infringement we will also deposit published papers on professional social media sites such as ResearchGate and LinkedIn to archive publications, providing easy access for interested parties.

Species and numbers of animals expected to be used

Mice: 1) Corneal wound-healing model=60 mice; and 2) Corneal-infection model=360 mice



Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Protocol-1: Corneal wound-healing model. BalB/C mouse is a widely used strain of animal for testing drugs for eye diseases as these animals have an excellent ability to heal corneal ulcers naturally.¹⁻⁶ For the treatment of corneal infection in humans, antibiotics are frequently applied as drops on the surface of eye at a wide range of concentration (0.1 to 5% w/v) at repeated intervals. Here, we will test the safety of peptide/antibiotic combination only on one eye of 8-10 weeks old adult mice in a similar fashion.

Under anaesthesia (both local and general), we will create the superficial wound on corneal surface (removal of central epithelial layers) using an appropriate published technique (e.g. ocular burr).³ Peptide (e.g. 0.01, 0.05, and 0.1% w/v) in combination with antibiotic (e.g. 1, 2, and 4% w/v) will be applied as drops on the surface of eye at an interval of 4 times/day (3 hours apart) for 3 days. Animals treated with clinical-grade antibiotic drops and sterile buffered saline will be used as control groups.

Healing of corneal wound will be checked every day up to 4 days using a clinical technique under mild anaesthesia (staining with a sterile sodium fluorescein dye). Images of stained cornea will be analysed using a specialised software (e.g. ImageJ) for measurement of corneal wound. At the end of day 4, animal will be killed to retrieve cornea tissue for laboratory-based analysis (assessment of inflammation).

Protocol-2: Corneal infection model. In humans, corneal infections are often regarded as emergency conditions and their treatment involves use of antibiotics as drops on the surface of eye at high concentrations in a repeated manner (*one-drop per hour for first 48 hours and then four times/day for next 3-7 days or until clearance of infection*). Corneal infection mouse model has been widely used to test the effectiveness of antibiotics and antibiotic-like drugs.^{7, 8} Our aim here is to utilise this model to test whether our peptide/antibiotic combination therapy is capable of clearing infection in the eye. Also, we will ascertain the key drug indices (pharmacokinetic parameters - important for designing future clinical studies) of peptide/antibiotic combination in this model. We have propose to use C57BL/6 strain of mouse for infection studies because they develop progressive infection similar to humans. We will set-up the corneal infection model using a well-characterised bug (*Pseudomonas aeruginosa*) as described in the literature.^{7, 8}

Under anaesthesia (general and topical), corneal surface will be wounded using a sterile 26-gauge needle (three parallel, 1-mm long superficial scratches). Bacteria (one million in 5 micro litre suspension) will be applied directly onto wounded cornea. Another drop of anaesthetic drug will be applied after the animal is awake. A single injection of pain killer (analgesic) will be given to alleviate any pain at day 0. *However, continuous and regular use of analgesic or topical anaesthetic is contradicted and will interfere with the outcomes of our proposed combination therapy.*

Six different combinations of peptide and antibiotic will be tested as eye-drops (starting 6 hours p.i.; 4 times/day (3-hours apart) for 4 days) in mouse with infected cornea. Levofloxacin and sterile buffered saline will be used as control drugs. Animals will be



monitored every 24 hours (**2-4 times/day**) for five days for clinical assessment of infection and general signs of well-being. All experiments will be performed in two groups in a staged manner: 1) Bacterial killing and 2) Advanced pharmacological assessment.

Group-1: As a first step, we will test the effectiveness of drugs in infection model. Mice will be killed at different days (day 1, 3, and 5) of treatment duration to assess how quickly the infection is cleared. We will specifically measure the amount of bacteria remaining (titer quantitation (CFU per gram)) in the cornea tissue, which will be retrieved from killed animals.

Group-2: For advanced pharmacological assessments, mice will be killed at different durations of treatment to retrieve bodily tissues. Cornea and serum will be analysed for measurement of free concentration of peptide and antibiotic using a specialised technique [e.g. Liquid chromatography- tandem mass spectrometry (LC-MS/MS) methodology (at the National Mass Spectrometry Facility, Swansea)]. Other bodily tissues from killed animal will also be collected and stored in deep freezers (-80C) for future analysis.

References:

Venkatesh M, Barathi VA, Leng-Goh ET, Anggara R et. al. Antimicrobial activity and cell selectivity of synthetic and biosynthetic cationic polymers. *Antimicrob Agents Chemother* 2017;61:e00469-17.

Shah M, Cabrera-Ghayouri S, Christie L, Held KS et. al. Translational preclinical pharmacologic disease models for ophthalmic drug development. *Pharma Res* 2019;36:58.

Kalha, S., Kuony, A., Michon, F. Corneal Epithelial Abrasion with Ocular Burr As a Model for Cornea Wound Healing. *J. Vis. Exp.* (137), e58071. (2018). doi:10.3791/58071.
Dua, HS., Gomes, JAP., Singh, A. Corneal epithelial wound healing. *Brit. J. Ophthal.* (1994). 78:401- 208.

Saika, S., Shiraishi, A., Saika, S., et al. Role of lumican in the corneal epithelium during wound healing. *J. Biol. Chem.* (2000). 275: 2607-2612. (doi: 10.1074/jbc.275.4.2607)

Huang Y-H, I C-C, Kuo C-H, Hsu Y-Y, Lee F-T, Shi G-Y, et al. Thrombomodulin Promotes Corneal Epithelial Wound Healing. *PLoS ONE.* (2015). 10(3): e0122491. doi: 10.1371/journal.pone.0122491

Hazlett LD, Ekanayaka SA, McClellan SA, Francis R. Glycyrrhizin Use for Multi-Drug Resistant *Pseudomonas aeruginosa*: In Vitro and In Vivo Studies. *Invest Ophthalmol Vis Sci* 2019;60:2978-2989.

Ekanayaka SA, McClellan SA, Barrett RP, Kharotia S, Hazlett LD. Glycyrrhizin Reduces HMGB1 and Bacterial Load in *Pseudomonas aeruginosa* Keratitis. *Invest Ophthalmol Vis Sci* 2016;57:5799-5809.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Protocol-1:



Mice with wounded cornea should only experience a mild level of discomfort (e.g. saline treatment group). Corneal wounds are naturally healed within 2-3 days.

If application of drops containing high concentration of peptide/antibiotic combination delays normal wound healing or show dense cloudiness (i.e., within 2 days), the mice is expected to experience a moderate level of discomfort (include hunched back, subdued and unresponsive behaviour, and greater than 15% bodyweight loss).

Non-healing or highly irritant cornea will show dense cloudiness covering the entire surface and this can be visualised cage-side without fluorescein staining. These animals will be taken off the study and culled as per Schedule 1 procedure.

The humane endpoint includes delayed healing of corneal wound leading to moderate discomfort, which includes hunched back, subdued and unresponsive behaviour, and greater than 15% bodyweight loss.

Protocol-2:

Corneal infection caused by bacteria are rapidly progressive, therefore, it is expected that corneal surface will start to show mild cloudiness starting as early as 12 hours post infection.

Mouse with a specific genetic features (C57BL/6 background) develops corneal infection similar to humans.

In this study, we will be using moderate infection protocol to set up a clinically-relevant pharmacological mouse model. The treatment will be stopped on day 5 and animals will be killed as per Schedule 1 procedure.

There is a possibility that up to 10% of mice (e.g. in saline control and low drug treatment groups) may develop corneal perforation (tiny hole) at day 5 post infection. Therefore, to minimise this incidence, we will monitor the animals every day (**2-4 times/day**).

Humane endpoint includes earliest clinical signs of corneal perforation and general signs of moderate discomfort.

Early clinical signs of corneal perforation include focal thinning of cornea without or with discharge (white to off-white).

General signs of reduced animal well-being include hunched back, subdued and unresponsive behaviour, and greater than 15% bodyweight 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 species)?

Protocol-1:

The corneal wound-healing is rapid and normally the superficial wound is repaired within 2-3 days.



Based on our *in-vitro* safety results, it is anticipated that about **10-15%** of experimental mice that were treated with higher concentrations of peptide/antibiotic will show the signs of moderate discomfort by day 4.

Protocol-2:

In group of experimental animals such as those treated with sterile saline solution (control group) and/or with low amounts of peptide/antibiotic, the infection will be progressive with mild cloudiness of cornea will be seen starting as early as 12 hours post-infection. In these group of mice, we expect to see common features of progressive infection such as cloudy cornea (covering entire corneal surface) with or without swelling/crater formation.

Based on previous experiences of our collaborator's laboratory, mouse with one eye infected with bacteria will experience moderate discomfort (at day 5 post-infection) with progressive infection. There is a possibility that **between 5-10% of mice in control group** may develop corneal perforation at day 5 post infection.

Note: We will monitor our mice every day (2-4 times per day) to minimise the incidences of corneal perforation. Animals showing early signs of worsening infection or corneal perforation such as focal thinning of cornea without or with discharge (white to off-white) will be immediately killed as per Schedule 1 procedure.

Similar to clinical cases, in group of mice that are responding to treatment, we do not expect to see any significant signs of discomfort and/or clinical signs of progressive infection.

Based on plate-based bacterial killing data, the infection is expected to be cleared within 48-to-72 hours post-treatment with alleviation of the complete or most disease symptoms within 5 days.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Wound-healing model: Our recent non-animal, laboratory-based study showed that the new peptide/antibiotic are safe and have ability to kill bugs. However, full testing requires a bodily (physiological) system, including a functional blood vessel support network. It is not feasible to produce an adequate model of this complex system using *non-living plate-based model*. Isolated *ex vivo* eye tests (using cadaver donor tissues) account for a degree of multicellular interplay and can provide limited biological data. Still these cadaver model unable to mimic the response of the complex biological (physiological and systemic) milieu, which are responsible for mediating wound healing. *In vitro* assays (plate-based) cannot adequately model the complete array of physiological healing responses to a wound important in ocular surface injuries. Therefore, proposed *animal* work is required to ascertain the safety of therapeutic compounds.



Corneal infection model: *In-vitro* and *ex-vivo* models of corneal infection are available. However, they do not recapitulate the *in-vivo* host responses to infection. Bacterial corneal infection is a progressive disease that triggers typical host responses such as inflammation, infiltration of defence cells through limbal region of cornea, and reduction of corneal nerves function and wound-healing processes. Therefore, testing of our proposed therapeutics in an *in-vivo* diseased environment will provide essential data and proof-of-principle for subsequent human-based clinical studies

What was your strategy for searching for non-animal alternatives?

In-vitro cell based infection model

Ex-vivo cadaver corneal tissue infection model

In-vitro cell-scratch model

Why were they not suitable?

The above-mentioned models may not recapitulate complete *living tissue* disease events (Infiltrating immune cells and bacteria interaction and the resultant tissue inflammation) and bodily environment (tears, blinking, and their impact on drug adsorption and availability).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 number of animals (sample size) was calculated based on published studies and our previous experiences.

Additionally, we have consulted a statistician based in our REDACTED to help with designing our animal studies.

All animals in proposed studies will be coded with a random number and studied in a masked fashion (where investigator performing experiments and those that are analysing data do not know about the treatment each animal have received). At the end of each experiment, the code will be revealed to perform statistical analysis between groups. This will allow us to reduce biased errors.

This is in accordance to human-based clinical studies and the ARRIVE guidelines for animal research.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Our published *in-vitro* results have demonstrated the superior safety and efficacy of test compounds and these results have formed the basis for the planned animal studies.



Proposed animal studies have been successfully funded by the Medical Research Council (MRC). This is a highly competitive award and undergo rigorous peer-review filtering.

All statistical analysis was prepared in consultation with a statistician based within the School of Medicine and these have been approved by the MRC peer-reviewers.

What other measures apart from good experimental design will you use to minimise numbers?

Our non-animal laboratory based results have successfully showed that the peptide/antibiotic combination is safe and effective. Having this key data have considerably helped us to reduce the total number of animals required for planned experiments in protocol 1 and 2.

As a first step, we will conduct pilot experiment using a small group of animals. This will optimise the key experimental procedures, ascertain the maximum amount of peptide/antibiotic that can be used safely, and will enable us to gather essential knowledge of disease progression in animals.

The results from experiments in protocol 1 is anticipated to provide essential clues to avoid application of drops (containing high amounts of peptide/antibiotic) that may cause corneal irritation in animals that will be used in protocol 2. This will also reduce the number of animals required in protocol 2.

Due to the costs of advanced analysis (e.g. quantification of free drug using LC-MS/MS methodology) and funding limits, we have only proposed to test the biological characteristics (e.g. pharmacokinetic parameters) of peptide/antibiotic in cornea and serum. We will also collect other bodily tissues from killed animals, which will prevent any duplication of studies and reduction of animal use for research in future. Tissues will be stored at deep freezers up to 2 years after completion of proposed study.

Table depicting the template of planned experiments under protocol 1. A similar plan will be followed for the protocol 2:

Wound-healing protocol		Gender		Group
Sample ID	Group			
1	CY	Female	AY	
2	BZ	Male	AZ	
3	CX	Female	AX	
4	BY	Male	BX	
5	AX	Female	Cont	
6	CZ	Male	AY	
7	AY	Female	BZ	
8	AZ	Female	CX	
9	BX	Male	CZ	
		Female	CY	
		Female	Cont	
		Male	BY	
		Male	BZ	
		Female	AX	
		Female	AZ	
		Female	CZ	
		Male	BX	
		Male	CX	
		Male	CY	

Experiment 1		
CAGE	GROUP	GENDER
1	AY	Female
	BX	
2	BZ	Female
	CX	
3	CY	Female
	Control	
4	BY	Female
	AX	
5	AZ	Female
	CZ	
6	AZ	Male
	AX	
7	Control	Male
	AY	
8	CZ	Male
	BY	
9	BZ	Male
	BX	
10	CX	Male
	CY	

Experiment 2		
CAGE	GROUP	GENDER
1	AY	Female
	BX	
2	BZ	Female
	CX	
3	CY	Female
	Control	
4	BY	Female
	AX	
5	AZ	Female
	CZ	
6	AZ	Male
	AX	
7	Control	Male
	AY	
8	CZ	Male
	BY	
9	BZ	Male
	BX	
10	CX	Male
	CY	

Experiment 3		
CAGE	GROUP	GENDER
1	AY	Female
	BX	
2	BZ	Female
	CX	
3	CY	Female
	Control	
4	BY	Female
	AX	
5	AZ	Female
	CZ	
6	AZ	Male
	AX	
7	Control	Male
	AY	
8	CZ	Male
	BY	
9	BZ	Male
	BX	
10	CX	Male
	CY	

Sample ID represents 9 different combinations of FK16 and Vancomycin

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the



procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Protocol 1 - Corneal wound-healing model: Mouse with superficially wounded cornea (corneal wound-healing model) naturally get healed within 2-3 days, therefore, should only experience a minimum level of discomfort. For animal welfare, we will be only wounding one eye for experimentation. All procedures will be performed under appropriate anaesthesia. To refine an ideal concentration, we will be testing a range of different amounts of peptide/antibiotics. Pilot experiment in a small group will establish the model system, standardise the key procedures, and refine the maximum amount of peptide/antibiotic that the animal could tolerate. The proposed concentrations was based on our *non- animal laboratory-based* safety and efficacy results. If the application of maximum concentration of combination therapeutics on wounded cornea delays normal wound healing then mice is expected to experience a moderate level of discomfort. However, the animals in experimental group that will show any signs of significant discomfort (at any time during study) will be immediately killed as per Schedule 1 killing procedure. Results from this model will provide us the information on the concentration of test combination therapy that is safe and do not delay wound-healing. This is to ascertain that the undue and significant discomfort to animals is prevented in subsequent planned studies in protocol 2.

Protocol 2 - Corneal infection model:

Mouse with one eye infected with bugs (optimised number of bacteria) is expected to experience a moderate level of discomfort at the peak of disease (at day 5 post-infection). Appropriate anaesthesia (general and topical) will be administered before wounding or infecting the animals' eyes to reduce the discomfort induced by the procedure. Another drop of anaesthetic drug will be applied when the animal is awake. A single dose of pain-killer can be provided to reduce any pain associated with the procedure. Animals treated with different concentration of drugs will be killed as per schedule 1 procedure for collection of eyes and blood.

Note: Continuous or regular use of anaesthetic/pain-killer are contraindicated for the management of corneal wound and infections because these have shown to worsen the healing process. *It is also noteworthy to mention that this same principle applies to patients who present with corneal infection in the clinical practice where anaesthetic drops are only used once during the initial corneal scraping for microbiological sampling and no further topical anaesthesia is continued based on the same reason.* Additionally, regular systemic analgesia may interfere with the biological outputs of planned studies.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice at mature age (8-10 weeks old) will provide us data needed for subsequent clinical studies in adult humans.

Body's defence system plays an important role in wound-healing and fight against infection therefore mice at immature life stage may not capitulate the clinical corneal disease.



Corneal infection is a progressive and active disease; therefore, it will be difficult to derive therapy data from terminally anaesthetised animals.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Only one eye of each animal will be wounded or infected and the other eye will remain untouched so that the impact on the general well-being of the animals is minimised to the best possible level. Corneal abrasion and infection will be performed under appropriate anaesthesia. Once the animal is awake after the procedure, one-drop of topical anaesthetic will be applied for post-procedure care. A single dose of pain-killer can be administered to manage post-injury pain at day 0 of the study. However, regular pain-killer or anaesthesia are contradicted and will interfere with the outcome of our therapeutics. We will utilise the appropriate clinical scoring and the NC3Rs mouse grimace scoring systems for post-infection or post-wounding assessment of animals for any significant discomfort. We will assess the mice daily (under the supervision of NVS) and if any study animal showing significant signs of discomfort will be killed as per ASPA Schedule 1 and tissues will be collected as appropriate.

The mice will be housed socially during maintenance, but will be housed individually or maximum two/cage (for those in protocol 2 - corneal infection model) in view of the risk of cross-infection. For animals in protocol 1, they will be housed in group of 2 as per block design.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Corneal wound-healing model:

Kalha, S., Kuony, A., Michon, F. Corneal epithelial abrasion with ocular burr as a model for cornea wound healing. *J. Vis. Exp.* (2018). 137: e58071. (doi:10.3791/58071).

Saika, S., Shiraishi, A., Saika, S., et al. Role of lumican in the corneal epithelium during wound healing. *J. Biol. Chem.* (2000). 275: 2607-2612. (doi: 10.1074/jbc.275.4.2607)

Huang Y-H, I C-C, Kuo C-H, et al. Thrombomodulin Promotes Corneal Epithelial Wound Healing. *PLoS ONE.* (2015). 10(3): e0122491. (doi: 10.1371/journal.pone.0122491)

Corneal infection model:

Parmar, A., Lakshminarayanan, R., Iyer, A., Mayandi, V., et al. Glycyrrhizin Use for Multi-Drug Resistant *Pseudomonas aeruginosa*: In Vitro and In Vivo Studies. *Invest Ophthalmol Vis Sci* 2019;60:2978-2989.

Ekanayaka SA, McClellan SA, Barrett RP, et al. Design and syntheses of highly potent teixobactin analogues against *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococci (VRE) in vitro and in vivo. *J. Med. Chem.* 2018;61:2009-2017. (doi: 10.1021/acs.jmedchem.7b01634).



Hill, L.J., Moakes, R.J.A., Vareechon, C., et al. Sustained release of decorin to the surface of the eye enables scarless corneal regeneration. *NPJ Reg. Med.* 2018; 3:23. (doi: 10.1038/s41536-018-0061-4).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

NC3Rs website (nc3rs.org.uk) will provide us the updates on the advances made in the 3Rs. Moreover, we will stay in regular contact with NACWO, NTO, and NC3Rs regional programme manager to receive any new updates related to 3Rs and implement them during the project.

We have access to the REDACTED-subscribed journal in the ophthalmology field, which will also keep us informed of new advances in studying preclinical models *i.e.*, experimental tools for studying how therapeutics kills bacteria in cornea of live animals. This will allow us to reduce the number of animals requested in this project.

Explain the choice of species and the related life stages

Mouse as a model system is key for drug discovery process and it is central to transitioning of potential drugs from laboratory-to-clinic. Planned studies to assess the safety, efficacy, and key biological factors (pharmacological parameters) in the proposed mouse model systems will inform whether drug concentration in cornea during disease condition is at safe levels and will be sufficient to clear infection. Additionally, outputs from mouse models will enable us to refine the dosages of drugs for advanced studies and reduce the costs that will incur for extensive human-based clinical trials.





130. Preventing Neonatal Infectious Arthritis in REDACTEDs: Sources, Transmission and Characterisation of *Streptococcus dysgalactiae*

Project duration

4 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

No answer provided

Animal types	Life stages
REDACTED	neonate, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The aim of the project is to identify methods to prevent a severe and common joint disease of new born REDACTEDs. We will study the bacteria which cause the disease, *Streptococcus dysgalactiae*, in order to identify sources and routes of transmission of infection from the mother and farm environment to the REDACTED. We will examine animal and farm level risk factors for disease. Our study will also contribute to separate project work to develop a vaccine against the disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?



Streptococcus dysgalactiae infectious arthritis (joint-ill) is a common condition of new born REDACTEDs that causes chronic pain and suffering to many REDACTEDs in the UK each year; as well as significant economic loss to the farming industry through REDACTED deaths and reduced growth rates. The disease will often occur despite farmers best efforts to prevent it. Therefore, to prevent "joint ill" and help improve REDACTED welfare this study will try to identify where the infections arise from, and how the bacteria reach the REDACTED in order to provide farmers with effective advice on how to prevent it. The study will also contribute to work on developing a vaccine against *Streptococcus dysgalactiae*.

What outputs do you think you will see at the end of this project?

Through a better understanding of *Streptococcus dysgalactiae* joint disease, transfer of new information and guidance to farmers and contributing to the development of a new vaccine, the project aims to benefit REDACTED welfare and contribute to a more sustainable and efficient REDACTED farming industry.

We plan to publish our work in publications in peer reviewed scientific journals. We plan to produce at least three scientific publications.

The findings of the study will be directly communicated to farmers in the form of updated guidance on prevention of *Streptococcus dysgalactiae* joint infections in REDACTEDs. This will be done through guidance documents, webinars, articles in farming press and on-line resources.

The outputs of the study will inform the development of a vaccine against *Streptococcus dysgalactiae* joint infections.

Streptococcus dysgalactiae bacteria are closely related to bacteria which cause human disease, and therefore knowledge of the bacteria gained here could also benefit human research.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The beneficiaries of the work are therefore animal welfare, the farming industry, the veterinary profession, and the scientific community. The outputs of the project are planned when the project is complete.

How will you maximise the outputs of your work?

The project is collaborating with the UK REDACTED industry to ensure maximum dissemination of the knowledge gained from the project to farmers and veterinarians.

The project is collaborating with existing work on vaccine development against *Streptococcus dysgalactiae*. An effective vaccine would be a substantial benefit in our ability to prevent *Streptococcus dysgalactiae* infection in REDACTEDs.

We will also create a biobank of samples and bacterial isolates obtained in the study for use in other research projects. Anonymised data will be archived for use in other studies.

Species and numbers of animals expected to be used



- REDACTED: 200 REDACTEDs and 700 REDACTED

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

There are two studies:-

In the first we will collect faecal, vaginal and skin swab samples and blood samples from REDACTED from farms affected by *Streptococcus dysgalactiae* joint infections to identify REDACTED that maybe carrying the infection and passing it onto their REDACTEDs. Any REDACTED that are positive for the infection and equal number of healthy animals will be resampled for the next two years in order to find out where the infection is harbouring and how long they carry it for.

In the second study we will collect blood, faeces and joint samples from REDACTEDs naturally affected by this *Streptococcus dysgalactiae* joint infection, after which we will give the REDACTEDs an appropriate treatment for the disease. We will collect blood and faeces samples from healthy REDACTEDs of the same age on the same farm for comparison. We will also collect environmental samples, as well as faeces, milk and vaginal samples from the mothers of diseased REDACTEDs and healthy control REDACTEDs so we may see whether the mothers are the source of infection to the REDACTED.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The REDACTEDs and REDACTED will experience brief pain or discomfort during the sampling which will last 10-20 minutes. They will remain on their own farms at all times, and diseased REDACTEDs will be provided with correct treatment for their 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 species)?

Severity is mild for all animals in the study.

What will happen to the animals at the end of the study?

- Rehomed

Replacement



State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We require to study *Streptococcus dysgalactiae* joint infections in REDACTEDs already naturally affected by the disease, their mothers and control animals for comparison.

What was your strategy for searching for non-animal alternatives?

There are no non-animal alternatives models for *Streptococcus dysgalactiae* joint infections in REDACTEDs. However, we have used a questionnaire of farmers to gather as much data as possible on disease prevalence and risk factors without using animal sampling.

Why were they not suitable?

There are no non-animal alternatives models for *Streptococcus dysgalactiae* joint infections in REDACTEDs.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used power calculations, estimates from previous studies and advice from other researchers in the field to calculate the sample sizes.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Advice from local statisticians, on line tools (Open Epi https://www.openepi.com/Menu/OE_Menu.htm) and advice from other researchers working in the field has been used.

What other measures apart from good experimental design will you use to minimise numbers?

The project sampling phase will run over three years so we will use data gathered in year one to determine if our calculations are accurate and if we are able to reduce our sample size. Furthermore in the longitudinal study all REDACTED on the farm will be sampled in year one but we are using a case control study design in years two and three to minimise animal numbers

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the



procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will study *Streptococcus dysgalactiae* joint disease in naturally occurring affected REDACTEDs.

We will collect the minimum number of samples and minimally invasive samples from these REDACTEDs and healthy control REDACTEDs and REDACTED from the same farms. Sampling will be brief (10-20 minutes). Local anaesthetic will be used where appropriate. All diseased animals will receive appropriate treatment for the disease. All animals will remain on the farm of origin.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

There are no animal models for this disease.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Local anaesthetic will be used for sampling from infected joints.

All animals will be monitored for at least 1 hour post sampling for any adverse effects, should these occur veterinary attention will be sought.

All diseased REDACTEDs will be treated appropriately with antibiotics.

Results of the study will be continuously monitored to ensure only the required number of animals will be sampled.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The study will be supervised at all times by an experienced farm animal veterinary surgeon.

All staff involved in farm sampling will be experienced in handling animals and trained personal licence holders.

ARRIVE guidelines will be used for study design and reporting.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The project licence holder will stay informed about advances in 3R's through engagement with the National Centre for Replacement Reduction and Refinement of Animals in Research Website and through seminars and information disseminated through the research institution where the project licence is held.



Explain the choice of species and the related life stages

The project will study the disease of young REDACTEDs *Streptococcus dysgalactiae* joint infections. We wish to collect samples from REDACTEDs already affected by the disease, their mothers and healthy control animals in order to understand the bacteria causing the disease; where the infection comes from; how it spreads to the REDACTED; and what farm management practices contribute to its occurrence.





131. Prevention of post-surgical adhesions

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant
Rats	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 is the aim of this project?

Post-operative adhesions are abnormal bonds formed between organs or tissues after surgical procedures, which is one of the most prevalent complications after surgery. This project aims to investigate the cellular and molecular mechanism by which post-operative peritoneal (intra-abdominal) and pericardial adhesions are formed and furthermore develop new treatments to prevent adhesion formation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Post-operative adhesions occur in more than 90% patients after surgical procedures. This can cause critical issues, including bowel obstruction, pain and female infertility. Currently, millions of patients suffer this complication all over the world. As the only effective



treatment, once developed, is invasive corrective surgery, prevention of this disorder is vital. However, current preventative treatments have limitations in the efficacy or availability. To develop innovative approaches, it is essential to further understand the mechanism underpinning the post-operative adhesion formation.

What outputs do you think you will see at the end of this project?

This project will develop pre-clinical evidence to validate new treatments to prevent post-operative adhesions, enabling further development of the innovative approaches forward to clinical application. In addition, this project will provide important new biomedical and scientific knowledge to understand the mechanism for adhesion formation and shed light on new biological insights, which will pave the way to future medical and scientific research. These pieces of new information will be presented at international conferences and published in academic journals.

There are also commercial opportunities associated with the innovative therapy for post-surgical adhesions (i.e. development of new drugs or new biomaterials). Therefore, this project potentially has a great impact on patients, doctors, scientists, the NHS and society. Thus, this project has great potential to help patients who have risks to develop post-surgical adhesions and save the cost to treat such patients.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Various people and sections will receive benefits from the outputs of this project.

Researchers (immediate): This basic and translational research project will provide a range of novel biological information regarding a mechanism by which post-operative adhesions are formed. This new scientific information will provide impact and benefits to researchers in a wide range of areas, including inflammation and immunology research. Also, through this research we will be able to provide high- standard training for the research staff and students involved in this project.

Patients, Clinicians and National Health Service (future): Post-surgical adhesions are one of the most important causes of complications following abdominal as well as heart surgery. This project will propose novel approaches for prevention of post-surgical adhesions, which will be further developed by subsequent pre-clinical and clinical studies. The new treatment proposed in this project is expected to effectively prevent such post-surgical adhesions, improving the outcome of surgeries, enhancing post- surgical quality of life of patients, and reducing labor and cost to maintain these patients. Thus, successful development of this treatment will offer great benefits on patients, clinicians and the National Health Service in the future.

Business and Society (immediate and future): Scientific success of this project will improve opportunities for advanced life science research training in cutting-edge technologies in the UK and attract international postgraduate and undergraduate students to our Institute as well as to the UK. Successful pre-clinical information obtained from this project will attract interest from commercial companies in progressing development of this innovative treatment towards clinical application, as this therapy possibly has a huge market. Through the effective dissemination of our results, the general public will be more aware of the benefits of developing and using a novel drug to treat human diseases. This will allow the full potential of such drug development to be recognized and will also attract large



numbers of younger generations to enter scientific disciplines as a career opportunity. These aspects will support the UK in maintaining its reputation in medical research.

How will you maximise the outputs of your work?

To achieve the maximum impact from this project, we will fulfill all aspects of communications, in cooperation with the College's departments for Research & Development, Intellectual Property, and Public Engagement, according to our dissemination and engagement strategies as follows.

Publication and presentation: As soon as sufficient data is obtained in the proposed project, it will be published in high-quality peer-reviewed academic journals subject to consideration of protection of their potential for intellectual property and commercial exploitation. Our publication strategy will be taken in accordance with the College policy on "open access" publishing. The obtained results will also be presented and discussed at scientific meetings/seminars (institutional, national and international). This will help to disseminate the obtained results to academics, clinicians, and companies in the relevant field. In addition, to raise awareness of the potential of our new therapeutic approach in the hospital, we will introduce our project to clinicians, nurses, and other hospital staff at clinical seminars/lectures and via hospital newsletters.

Data sharing: Data produced in the proposed project will be preserved within our Institute for sharing among the researchers according to our institutional policy on data sharing and preservation. Raw data will be retained and available for possible review/audit, in digital format and as original materials. We will encourage collaborations (internal, external and international) via our website with a link to this project and through communications during the conferences.

Exploitation: Obtained results may include patentable intellectual property. Intellectual Property Department in our institute will help handle intellectual property issues, licensing, and commercialization of the exploitable results produced in this project.

Public engagement: Our REDACTED, School and Institute all have active public engagement programmes. Results produced are open to the public on their homepages and will be published in the Bulletin/Newsletter and REDACTED publications. Our publications contain special lay sections to make data accessible to public. If appropriate, the results of this project will be disseminated by media broadcast and newspapers. These engagements will collectively enhance public awareness of the importance of this project. In addition, our institute, particularly the Public Engagement Department, runs a number of outreach projects, including annual science festival, designed to reach local school pupils to raise their career aspirations through activities that include science experiments, work experience placements, visits to the College and talks from international leaders in medical research. Our project will be introduced during such public activities.

Business: This project will pre-clinically develop an innovative therapy for prevention of post- surgical adhesions. Thus, there is a good opportunity for business development, potentially attracting the interest of biomedical ventures or mega-pharm. Through our dissemination strategy above, we will maximize such an opportunity. I will be responsible for identifying additional intellectual properties and establishing appropriate protection of the data from this project before public dissemination.

Clinical medicine: Although the knowledge obtained in this project cannot be directly applied to the bedside, this project is one of the major steps to reach the ultimate goal of



the establishment of a novel therapy for prevention of post-surgical adhesions. To raise awareness of the potential of our new therapeutic approach in hospitals, we will disseminate the progress of the project among doctors and other hospital staff through clinical seminars/lectures/conferences and hospital newsletters.

Species and numbers of animals expected to be used

- Mice: 2000
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The adult animal will receive a peritoneal or pericardial adhesion-inducing surgery with one of the treatments to regulate adhesion formation (either prior to or at the time of the operation). Subsequently, animals will be followed up for adhesion formation for up to 90 days (commonly within 4 weeks). Then, animals will be humanely sacrificed so that the degree of adhesion formation can be assessed and tissues/organs are collected for histological and biochemical investigations.

We will produce genetically altered animals, which do not exhibit any harmful phenotype. We will also produce bone marrow chimera mice through irradiation and bone marrow transplantation.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Animals are expected to recover uneventfully from any procedures in our project. No death is predicted by the protocols, and animals are likely to experience discomfort and pain of the mild-moderate severity temporarily during the immediate post-procedure period when the adverse effects will be limited with our countermeasures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Up to the moderate severity in all animal types used.

What will happen to the animals at the end of the study?

- Killed

Replacement



State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The proposed project aims to develop a new treatment to prevent post-operative adhesions. In order to evaluate therapeutic effects of the treatment, measurements of the degree of post-surgical adhesions after the treatment are essential. The process to form post-surgical adhesions is extremely complicated, involving multiple cell types (both locally resident and recruited) as well as many molecules and signalling pathways, which cannot be mimicked without the use of animals.

What was your strategy for searching for non-animal alternatives?

We have considered a potential use of computer-based systems, lower organisms and embryo stages, 2-dimensional and 3-dimensional culture of cells relevant to adhesion formation including peritoneal and pericardial cells. We have also considered the possible use of culture of peritoneal/pericardial tissue and organs.

Why were they not suitable?

Because any non-animal alternatives cannot appropriately represent complex in vivo processes associated with post-surgical peritoneal or pericardial adhesions, which include multiple cell types and cytokines/chemokines/growth factors. In addition, inflammation and fibrin formation/lysis, both of which are regulated by multiple cell types and factors, are tightly related in the mechanism of adhesion formation post-surgery. These work together, mutually affecting each other, toward adhesion formation as a whole. Only living mammals can be meaningful models that mimic the clinical scenario of post-operative adhesion formation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 methods, experimental designs, and methods for analysis of the results have been discussed with the Statistical Service Unit in our Institute. The design of individual experiments generally involves factorial designs, which maximizes the information obtained from the minimum resource. Some of measures are essentially qualitative; others are quantitative, for which statistical analysis may be appropriate.

For the quantitative experiments, sample sizes are set using power analysis. Generally, the significance level will be 5%, and the power 80%. For example, in a four-group experiment, if the least practicable difference between groups is chosen to be 25% and if the coefficient of variation is estimated to be 15%, then about 7 animals per group would be able to give a solid conclusion.



Having said this, the exact numbers of animals required will vary according to the particular experimental model/treatment and the estimate of the coefficient of variation, etc. Such estimation may be difficult from only in vitro studies. In this case, we will conduct an in vivo pilot study to estimate the degree and variation of the effect, using which the accurate group size in each study will be determined.

For the qualitative experiments, the number of animals required will be the minimum necessary to provide an adequate description.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Experiments were in principal designed by referencing to the NC3Rs Experimental Design Assistant. It is known that the degree of post-surgical adhesions is affected by surgical procedures. Good Practice should prevent, or at least minimise the introduction of bias into the experiments. This will, in turn, help significant reduction of the animal numbers used in this project. Accordingly, individual experiments will include:

Randomisation of treatment or control

Allocation concealment

Blinded assessment of outcome

Explicit inclusion and exclusion criteria

Sample size calculations done before the experiment

Monitoring and controlling of blood pressure and temperature during surgery

Stable depth of anaesthesia

For each and every experiment, as part of good laboratory practice, we write an experimental protocol which includes:

A statement of the objective(s)

A description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animal/group), and the experimental material.

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).

What other measures apart from good experimental design will you use to minimise numbers?

In principal, we will refer to the NC3Rs Experimental Design Assistant. Animals will be bred efficiently using a Standard Operational Protocol in our Biological Sciences Unit. In vitro pilot studies will be carried out to exclude less-promising approaches to regulate post-operative adhesions. All useful tissues/organs will be collected from each animal to reduce the number of animals required. Tissues/organs collected will be used to assess as many biochemical parameters as possible, which will also help to minimize 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use rats and mice, which are the most suitable for this basic/pre-clinical investigation; basic pathophysiology on post-surgical adhesions is sufficiently compatible between human and rodents. In addition, the rodent models allowed the use of genetically altered animals or bone marrow chimera model.

Adhesion-inducing operation, which is used in this project, is the only appropriate experimental model to represent the clinical process of post-surgical adhesion formation. As the proposed project requires evaluation of post-surgical adhesions in clinically relevant settings, this model is needed. It is a fact that all previous reports have used this model to produce post-surgical peritoneal or pericardial adhesions.

The adhesion-inducing operations, either peritoneal or pericardial, are recovery surgeries and are classified to be “moderate severity”. Following this surgery, animals usually behave normally without any symptom. Appropriate analgesic regimes will be used during the surgery and also prolonged as necessary in order to minimise the pain, suffering and distress to the animals.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

There is no established model of post-surgical adhesions in less sentient animals due to technical difficulty. In addition, the basic pathophysiology on post-surgical adhesions is likely to be different between human and less sentient animals. It is needed to study living animals in order to mimic the complex, long-term process of post-surgical adhesion formation. Having a similar anatomy of intra- abdominal organs and heart/lungs is essential, while this is not available in less sentient animals.

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We have used the procedures for the last 5 years, which have been well established and refined.

However, we will continue our effort to further refine the procedures to minimize the animal suffering. This may be achieved by:

the use of newly-introduced, more effective analgesic regimen and/or antibiotics. This will be regularly discussed with the NVS.

less-invasive surgery, including minimizing the incision and reducing the surgical time.
improved post-operative observation procedures.

minimally invasive ways to assess the progression of adhesions, including advanced imaging.



What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

I will regularly refer to the Home Office homepage and documents, NC3R homepage and documents, and ARRIVE or PREPARE guidelines. This ensures full transparency in reporting all the in vivo experimental data.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I will stay informed about the latest advances in the 3Rs through information/newsletters from the Home office and NC3Rs, attendance at relevant academic conferences and regular literature search. Identified new methods/protocols will be discussed with our Biological Sciences Unit staff, NVS and Home Office Inspector for the implementation.

Explain the choice of species and the related life stages

The proposed project requires evaluation of post-surgical adhesions in clinically relevant settings. Adhesion-inducing surgery is the only appropriate experimental model to represent such a clinical process. We will use adult rats and mice, which are the most suitable for this basic/pre-clinical investigation; basic pathophysiology on post-surgical adhesions is sufficiently compatible between human and rodents. Lower species are not suitable to this aim. In the rodent models, we will be able to use genetically altered animals and bone marrow chimera animals (animals whose bone marrow is replaced with other animal's bone marrow that has specific features). The use of these animals enable in-depth investigation of molecular and cellular mechanism for post-surgical adhesions.





132. Production of antisera and immune cells in fish

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
All fish species	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 is the aim of this project?

The aim of this project is to generate antisera and immune cells from fish for research and diagnostic purposes.

This will support and improve the maintenance of aquatic biosecurity for farmed and wild fish as part of compliance with national, EU and international legislation regarding aquatic disease control.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

With the decline in wild fish stocks, aquaculture, the fastest growing food-producing sector globally, is increasingly critical to future global food security. Infectious diseases causing significant losses to farmed fish continue to be a major constraint impacting on both economic resources and animal welfare. The sera generated under this project will be used in characterisation and improved understanding of aquatic diseases. The sera will be



used in the development of diagnostic assays and in the regulatory testing of starting materials feeding into vaccine development for serious diseases of cultured and wild fish.

What outputs do you think you will see at the end of this project?

A successful epidemiology programme and accurate diagnostics are essential to prevent the introduction and control the spread of aquatic disease. The outputs from the project will improve our ability to detect and identify pathogens, to support UK aquatic biosecurity and comply with international legislation on aquatic disease. The availability of specific immunoreagents is vital to development, validation and implementation of tests designed to trace and control any spread of infection. Availability of reagents for pathogens present in the UK is also of considerable importance to follow the development or introduction of new strains of significant bacterial and viral pathogens that are defined by serotype.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The principal output will be evidence for policy and regulation. The improvement of our current serological tests and subsequent use in refined epidemiological surveys will aid the development of policy and regulations at both national and international levels, particularly in relation to the risk of exotic or emerging pathogens to native species in the wild and farmed environments. Outputs are typically in the form of advice reports, conference proceedings and peer-reviewed papers.

How will you maximise the outputs of your work?

Improved serological assays will be made public and may be suitable for commercialisation and uptake by the wider scientific/public community.

Additional benefits may be realised by use of these sera in fundamental and applied (translational) microbiological research and development of effective treatments.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Use of fish will be solely for the production of polyclonal antisera and immune cells.

Fish under procedure will receive a maximum of 5 injections of the antigen and adjuvant mix with an interval of approximately 500 DD between immunisations; and blood samples might be taken to assess the immunoglobulin titres with a maximum volume of blood permitted of 0.4% of body weight per sample, and no more than 15% of total blood volume over any four-week period before the final blood harvest.



The antigen may be used with adjuvants. The adjuvant programme will be selected depending on the study, with as minimal local reaction as possible whilst remaining effective. Freund's Complete Adjuvant (FCA) can be used only on the primary injection. For a fish weighing more than 50 g, the maximum volume injected per immunization by IM is 100 μ L of the antigen/adjuvant emulsion in each of two sites. The maximum volume by IP is 100 μ L of the emulsion in one site. Stable emulsions should be used with no more than 50% FCA mixed with antigen in aqueous solution. The antigen/adjuvant mixture will not exceed 100 μ L in fish <50g or 200 μ L in fish >50g. Alternative mineral oil vaccinations will also be considered (e.g. SEPPIC Montanide ISA) where evidence of reduced tissue reaction is provided. General anaesthesia with recovery will be administered for the immunisation injection.

Immunisation by the oral/immersion route is preferred when fish size (i.e. small fish \leq 20g) and antigen nature allows it. Antigen will be administered either by bath or as a food additive.

Marking (optional) can take place together with pre-immunisation blood sample. May be carried out by visible implant elastomers (VIE), passive integrated transponder (PIT) tags or suitable alternative methods. Marking can be repeated if loss of the tag is observed alongside booster immunisation or blood sampling. The most appropriate tag will be chosen depending upon the fish species and animal size.

The procedure described above will follow a full formal Study Plan. These Study Plans are developed following PREPARE guidelines (Smith et al., 2018) and require the consideration and approval of the AWERB and the Project Licence holder, before any experimental work commences. Copies of all approved Study Plans are filed in the Experimental Facility records area, by the Study Director, and in the electronic archive where they are readily available.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Bath immunisation in fish may cause loss of appetite for a short time.

Intramuscular (IM) or intraperitoneal (IP) immunised fish may develop lesions in the site of injection due the use of oily based adjuvants, especially when using Freund's Complete Adjuvant (FCA).

In IP injected fish, the most common type of reaction is a localised fibrinous peritonitis in the area adjacent to the injection site. Fibrinous strands typically form adhesions between the ventral body wall, spleen and pyloric ceca which may lead to mild and moderate lesions. IM immunisations are less common in fish, as the oil adjuvants can lead to the development of granulomatous inflammatory infiltrates. The IP route of injection is preferred over IM, except when using DNA-plasmids for the immunisation, where the IM route of infection has been shown to be more efficient.

Adverse effects due to the nature of the antigen:

Anaphylactic shock- Unlikely. It will be avoided by suministrating the smaller amount of antigen/adjuvant possible and by following the recommended doses of immunisation.



Inactivated pathogen used as immunogens may cause an infection – Unlikely, pathogens will be inactivated. When possible, antigen inactivation will be confirmed *in vitro* prior to immunisation (e.g. by growth in cell culture).

Adverse effects due to the nature of the adjuvant:

Granulomas caused by infection or adjuvant – Antigen will normally be combined with an adjuvant in order to enhance the immune response. Adjuvants will be chosen to stimulate the required antibody response whilst minimising local tissue damage. Selection will depend upon the route of administration, the nature of the antigen and previous experience. In order to minimise any adverse effects of immunisation, the sites of injection will be well separated, and regular inspection will be made. If any fish show persistent or extensive lesions or signs of distress, the monitoring frequency will be increased. The advice of the NVS and the NACWO will be taken on whether the animal needs to be humanely killed by S1M or the non-S1M method as follows: surgical anaesthesia followed by the collection of blood sufficient to result in exsanguination, followed by removal or destruction of the brain.

Table 1. List of possible adverse effects, likelihood, estimated duration, controls and humane endpoints.

	Adverse effects (What could go wrong)	Indicators (How we will recognise it)	Checks (How we will monitor it)	Estimated duration	Likelihood	Controls (How we will prevent or ameliorate harms)	Humane endpoints
Procedure-related	Injuries from anaesthesia, vaccination and bleeding procedures	Skin lesions, bruising/scale loss	Visual checks	< 24 hr	Likely- max: 40% of IM injected fish; less than 10% of IP injected fish	Appropriate procedures, training and skilled operators	Individual euthanasia if open/haemorrhagic lesions.
	Systemic allergic reaction (anaphylaxis)	Abnormal behaviour (extreme ventilation, unusual positioning, loss of equilibrium)	visual checks	< 4 hr	Unlikely	Correct dosage and use of adjuvants	Individual / population euthanasia if abnormal fish behaviour persists
	Side effects of vaccine components (adjuvants)	Lesions at injection site, temporary reduced appetite	Visual checks	< 3 days	Unlikely	Good hygiene during vaccination preparation and procedures, controlled dosage type of adjuvants; increased frequency of monitoring	Individual euthanasia if open/haemorrhagic lesions, or prolonged inappetence leading to emaciation
	Pathogenic disease (incomplete antigen inactivation)	Clinical signs of illness	Visual checks	< 2 days	Unlikely	Confirm effective inactivation, short term temperature manipulation outside of permissive temperatures	Individual euthanasia at confirmation of pathogen related clinical sign >mild. Population euthanasia if therapeutic treatments unsuccessful/ unavailable.
Not procedure-related	Aggressive interactions and cannibalism	Physical injuries to fins, eyes, skin. Observations of aggression/cannibalism	Visual checks	< 3 days	Unlikely	Grading for similar size; Ensuring appropriate feed ration; Maintain environmental conditions (e.g. water flow, fish density) known to minimise risk; Tank enrichment	Individual euthanasia if injuries considered > mild (e.g. open / haemorrhagic lesions).
	Poor water quality	Abnormal fish behaviour	Visual checks + routine and responsive water quality monitoring;	< 2 days	Unlikely	Stock at and maintain fish biomass within appropriate loading rate (kg/m ³ /L, i.e. biomass per inflow) for temperature and fish size. Increase water/air in flow rates.	Individual / population euthanasia if abnormal fish behaviour persists after mitigation actions taken.
	Pathogenic disease (unrelated to vaccine antigen)	Clinical signs of ill-health	Visual checks	< 2 days	Unlikely	Prior health screen samples; use of in house spf fish, prevention of pathogen presence - via prior quarantine, prophylactic and therapeutic treatments as advised by NVS, biosecure working practices and facility; maintenance of tank hygiene	Individual euthanasia at confirmation of pathogen related clinical sign >mild. Population euthanasia if therapeutic treatments unsuccessful/ unavailable, or >25% of population affected.
	Non-pathogenic disease	Clinical signs of ill-health; poor performance	Visual checks; Growth monitoring	< 3 days	Unlikely	Checks on performance during quarantine and acclimation. Provision of complete commercial diets. Obtaining fish from proven reliable suppliers.	Individual euthanasia at confirmation of clinical sign > mild. Population euthanasia if >10% of population affected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

40% of IM immunised fish may reach MODERATE in recognition that the occasional use of Freund’s Complete Adjuvant (FCA) may induce localised granulomas.



10% of IP immunised fish may reach MODERATE due the development of moderate lesions associated with adhesions in the ventral body wall.

60% of IM and 90% of IP immunised fish should not experience severity above MILD.

100% of fish immunised solely by the oral/immersion route should not experience severity above MILD.

100% of fish used to maintain the stock density will not experience severity above SUB-THRESHOLD.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 license aims to improve non-lethal immunological tests currently used in epidemiological surveys. In contrast to molecular tests, which inform the presence or absence of a given pathogen, serological tests detect previous pathogen exposure and are needed for the designation of disease-free areas for trade purposes (visit OIE <http://www.oie.int/animal-health-in-the-world/official-disease-status/>) and inform on pathogen introduction and epidemiological surveys.

What was your strategy for searching for non-animal alternatives?

In vitro culture will be used to produce the antigen (i.e. use of susceptible fish cell lines for culturing virus). *In vitro* methods will also be used to confirm antigen inactivation prior to immunisation.

Alternative methods for polyclonal antibody production have been considered, such as recombinant antibody phage display. However, due the nature of the autologous antisera reference test material required and neutralizing capability of the polyclonal antibody, the use of animals is required. During this licence, advances in neutralising antisera production using non-animal platforms will be closely followed, as well as attendance at specialised workshops and networking on non-animal-based antibody production.

Why were they not suitable?

Suitable synthetic non-animal alternatives do not currently produce antibodies with the range of specificity and affinity required in the assays these sera will be used for. For serological test development known positive controlled sera from the host species (fish species) are 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?

Power calculations are not needed for this license. The number of animals used in each assay will be the minimum required to produce the volume of antiserum needed and will depend on the species chosen and the antisera titres.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Control fish (non-immunised fish) will not be used in this licence as the immune status of each individual animal will be determined in the pre-immunisation blood sample. However, fish stocking densities and population size are necessary considerations to ensure the expression of normal feeding and social (e.g. schooling) behaviour and to minimise aggressive behaviour. The minimum number of fish per tank will depend on the fish species, animal size, and the species needs following advice from fish husbandry experts. Experimental facilities offer a range of flexible tank sizes to minimise animal numbers while enabling basic social behaviours. Taking into account individual variation in response, a maximum of 400 fish over 5 years is expected to maintain stock density and produce the volume of antiserum required. Depending on the titre of the antisera, a single batch of antiserum appropriately stored will last for many years and may be shared with other laboratories in the same research field. Heterologous antisera against a small number of fish pathogens and anti-species immunoglobulins are commercially available. Where these have been demonstrated to be suitable for our purposes, these will be purchased.

What other measures apart from good experimental design will you use to minimise numbers?

Every effort is made to reduce numbers of fish used to a minimum, incorporating animal husbandry expert advice on fish social needs and AWERB scrutiny on each study plan. Additionally, within the constraints of available tanks sizes and appropriate animal numbers required for acceptable stocking densities and with respect to individual variation in immune response, large fish will be used to generate larger and longer lasting volumes of sera from fewer 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?



Specific pathogen-free (SPF) common carp, Atlantic salmon and rainbow trout will be used for the antisera production under this License. Those species are the most relevant for UK aquaculture industry. The laboratory has more than 50 years of husbandry experience with these species. Variation in response between animals is minimised by size grading, and prior group holding in common conditions. Consideration is given to providing an appropriate environment, including enrichment such as shading and/or refuges, during experiments. Stock density, water current and conspecifics that promote social behaviour, plus nutritionally complete diets are carefully considered throughout the animal holding. Fish are stocked into experimental tanks by staff experienced in fish handling and are routinely acclimated after stocking prior to study initiation.

The adjuvant selected for the antigen emulsion will be the one that causes least discomfort but elicits the desired immune response. The selection of the adjuvant will be carefully justified in a Study Plan after discussion with the NACWO, NVS and the Licence Holder. Consideration will also be given to the route of administration when deciding the fish species and size. When possible, immunisation by immersion/oral route will be selected, to minimize the severity of the procedure (i.e. immunisation injections will not be needed).

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Rabbits and mice are often the standard laboratory animals used for the generation of polyclonal and monoclonal pathogen-specific antibodies. However:

Fish are less sentient than mammals, as they do not have the extensive cerebral cortex seen in the forebrain of mammals.

Major differences in Ig isotypes and performance within lower and higher vertebrates exist. For this reason, for the planned research into fish diseases, mammalian antibodies are inadequate.

The use of fish larvae for immunisation is not an alternative for the generation of positive sera as they lack a full developed humoral system. Furthermore, the likely yield of serum from such immature forms would be inadequately small.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Where possible, we will administer antigen by the mildest possible route - e.g. orally where possible, and only use parenteral routes where there is scientific evidence that the oral or immersion routes would be unsuccessful. Intraperitoneal (IP) injections will be preferred to intramuscular (IM).

Changes to the diet prior to oral immunisation, to mitigate any adverse impacts on feeding (e.g. palatability adaptation) will be discussed with the NACWO and NVS and stated in the Study Plan.

General anaesthesia will be bath administered prior to IP or IM injection and prior to blood sampling. Experienced PIL holders will conduct the anaesthesia following standard operating procedures.



Fish under procedure will be monitored via direct visual checks of condition and behaviour by both husbandry staff (during feeding and tank cleaning) and experienced PILs (at least daily) using standardised in-house scores sheets. The frequency of direct PIL checks will increase if adverse effects are present. If clinical signs associated with suspected infection with pathogen in use is observed (see Table 1) the animal will be schedule 1 method (S1M) terminated or killed by the humane non-S1M as follows: surgical anaesthesia followed of collection of blood sufficient to result in exsanguination followed by removal or destruction of the brain.

Direct visual checks will be accompanied by video observations using underwater cameras mounted within tanks. Water temperatures are recorded automatically and maintained at $\pm 0.2^{\circ}\text{C}$. Fish are normally fed at least once per day by hand. Hand-feeding means that fish feeding behaviour (a good indication of fish welfare) is better observed than if mechanically fed. Water replacement is normally maintained at D5 changes per day. Call out alarms are installed at the REDACTED facility which activates if pre-set parameters are exceeded.

Consideration is given to providing an appropriate environment, including enrichment such as shading and/or refuges, during experiments. Stock density, water current and conspecifics that promote social behaviour, plus nutritionally complete diets are carefully considered throughout the animal holding. Fish are stocked into experimental tanks by staff experienced in fish handling and are routinely acclimated after stocking prior to study initiation.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Protocols used will follow the Home Office guidance: "Antibody Production, Principles for Protocols of Minimal Severity". Fish blood samples will follow the guidance from Canada Department for Fisheries and Oceans, Canadian Council on Animal Care, September 2004. 4.0 Blood sampling of Finfish.1-15.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

During this licence, advances in neutralising antisera production using non-animal platforms will be closely followed, as well as attendance at specialised workshops and networking on non-animal-based antibody production.

Explain the choice of species and the related life stages

Rabbits and mice are often the standard laboratory animals used for the generation of polyclonal and monoclonal pathogen-specific antibodies. However, in recent years a greater understanding of humoral responses in fish has highlighted major differences in Ig isotypes and performance. For this reason, for the planned research into fish diseases, mammalian antibodies are inadequate. Therefore, after confirming materials are not available via other research groups or commercially, this licence seeks authority to produce autologous immune sera in fish to improve non-lethal immunological tests.

When possible, specific pathogen free fish will be used for the antisera production under this License. Atlantic salmon, rainbow trout and common carp are the most relevant for UK aquaculture industry. The laboratory has more than 50 years of combined husbandry



experience with these species. Variation in response between animals is minimised by size grading, and prior group holding in common conditions.

We will preferentially use these species for the generation of control sera for the development of immunological-based diagnostic tests. However, wild caught-fish will be used in the absence of commercially available stocks (e.g. European eel).

To generate positive immune sera, the fish need to be capable of generating a strong measurable humoral response. The first appearance of IgM in lymphocytes varies considerably among fish species. The use of larvae for immunization is not an alternative for the generation of positive sera as they lack a full developed humoral system. Furthermore, the likely yield of serum from such immature forms would be inadequately small. Thus, this license seeks authority for the use of juvenile and adult fish for the collection of large amounts of control sera.





133. Radiation-induced intestinal carcinogenesis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Mice	adult, 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 is the aim of this project?

To study the effects of exposure to x-rays on the induction of intestinal carcinogenesis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The International Commission on Radiological Protection (ICRP) is an independent Registered Charity, established to advance for the public benefit the science of radiological protection, in particular by providing recommendations and guidance on all aspects of protection against ionising radiation (ICRP.org). ICRP have a long history of use of data from large scale radiation accidents, including those from the Japanese atomic bomb survivors where alimentary tract cancers were shown to be an important component of the overall risk of late effects following high dose-rate radiation exposure. Additional epidemiological studies e.g. Ozasa et al, IJRB 95:879-891 2019, review epidemiological studies of individuals who were exposed to atomic bomb radiation and their children who were conceived after parental exposure to radiation have contributed a large amount in terms of our understanding of the late health effects of atomic bomb radiation and it's transgenerational effects. The epidemiological studies, in particular, have demonstrated



increased radiation risks for malignant diseases among survivors including those exposed *in utero*, and possible risks for some non-cancer diseases.

In their publications the ICRP describe in detail the process for use of epidemiological data, including a-bomb survivor studies, in parallel with animal experiments, in particular the doubling dose from the megamouse studies to come to conclusions regarding the population based risks of radiation exposure in terms of cancer and genetic (heritable) effects. In brief, the incidence data and the background levels of disease are used to define the doubling dose, which is then converted to risk per unit dose. The risks from different types of disease are summed and averaged to calculate overall detriment. For induction of all cancers, the current risk is calculated to be 5.5% per Gray for the whole population, and for heritable effects the risk is of the order of 0.2% per Gray. However, ICRP are explicit in that these figures are uncertain, and that population based risks cannot be applied to individuals, and, further make a clear case for further work to more fully characterise population based and individuals risks in terms of specific cancer types.

The work undertaken on this Project Licence will be the completion of an experiment currently in progress where genetically predisposed mice are being exposed to one or two doses of x-rays at different ages. The completion of the study was delayed due to concentrating on an acute study using gamma irradiation and breeding issues with the C57BL/6 *Apc^{Min/+}* colony. These mice have been successfully bred at this establishment for over 20 years and these issues have now been resolved. The completion of this study will give information on the effectiveness of a split-dose of radiation on the induction of intestinal tumours i.e. are the doses additive and what are the effects of irradiation at different ages on tumour number. Pathological examination of selected tumours from each group will be undertaken and DNA extracted from the tumour material for use in molecular studies comparing gene losses in tumours from the different experimental groups.

What outputs do you think you will see at the end of this project?

The CONCERT and MELODI SRA and roadmaps identify a number of key uncertainties in radiological protection which are of high priority for research. These include: (i) the risks posed by low doses of radiation, (ii) the effects of different types of radiation, (iii) the impact of age at irradiation, (iv) the role and identity of genetic factors in radiation sensitivity and (v) the importance of interactions between radiation and other contributory risk factors for radiogenic diseases.

The studies proposed in this Project Licence use a genetically altered mouse model of gastrointestinal cancer and will provide information on the relative biological effectiveness of x-rays given at two different times for *in vivo* gastrointestinal cancer induction (addressing uncertainties (ii) and (iii)) and on the gene losses in the tumours induced (addressing uncertainties (iv) and (v)). As such, the proposed project is of high priority for the UK Department of Health which sets research priorities in the UK. The information obtained in the proposed project will feed into the development of refined models for cancer risk estimation in human populations.

As detailed above, the results from animal studies are crucial to support low dose epidemiological studies, to provide clear scientific understanding to underpin appropriate and thus effective radiation protection. Ultimately, the project results might also inform personalised radiation protection which could eventually be implemented on the basis of genetic risk as well as other factors.



What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The results from previous animal studies examining the induction of intestinal tumours by ionising radiation and the *Apc^{Min/+}* mouse model have already contributed to the evidence base for providing advice and guidance to international organisations (e.g. The International Commission on Radiological Protection (ICRP), International Atomic Energy Authority (IAEA)), relevant government agencies (e.g. Department of Health), and the nuclear industry.

There is clear epidemiological evidence from the Atomic Bomb survivors that age at irradiation is an important factor, the younger you are at exposure, the longer you have to live leading to a greater risk of cancer. Current work is the evaluation how epidemiological studies of background radiation and childhood cancer can improve the understanding of the effects of low-dose radiation (<https://www.ncbi.nlm.nih.gov/pubmed/31751953>). ICRP have also recently highlighted there is a lack of evidence on these effect <https://www.icrp.org/publication.asp?id=ICRP%20Publication%2012>.

During the course of this project, a greater knowledge of the effects of cumulative exposure administered as two separate doses given at neonatal ages (2 and 10 days old) combined with irradiation as young adults and adults (30, 45 and 90 days old) and aged adults (180 days old from a previous experiment) on radiation-induced intestinal carcinogenesis will be obtained. This will give information on the effects of irradiation at different ages and if a second, later irradiation has any effect on tumorigenesis i.e. are the effects additive?

In the longer term, molecular biological analysis of the tumours will provide information on the genes and gene losses involved in tumour formation which will further inform radiation protection legislation and guidelines and be useful in addressing the identity of genetic factors in radiation sensitivity in people with *APC*.

The results from animal studies such as those undertaken in this project are crucial to support low dose epidemiological studies, to provide clear scientific understanding to underpin appropriate and thus effective radiation protection. Ultimately, in the future, the project results may also inform personalised radiation protection which could eventually be implemented on the basis of genetic risk as well as other factors.

How will you maximise the outputs of your work?

The results from these studies will be presented at meetings and conferences (for instance the annual European Radiation Protection Week) and submitted for publication in the open scientific literature and thus add to the knowledge base available to researchers worldwide and used by ICRP, UNSCEAR and WHO in their future radiation protection guidelines. The raw data from individual tumour counts and size data will be placed on STORE (http://storedb.org/store_v3/) to allow future use by other researchers and avoid repetition of animal experimentation.

Species and numbers of animals expected to be used

- Mice: Inbred and genetically altered mice, 1,250 animals during the project licence.

Predicted harms



Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mice used for breeding will be mated at 5 or 6 weeks of age and kept for their useful breeding lifespan (usually 3-4 litters).

Mice exposed to x-rays as neonatal animals will be placed in Corex boxes in their nesting material (as they are not expected to move around the cage) and adult mice will be placed in the boxes without any nesting material as this allows them to run around freely. The mice will be exposed to either 2.5 Gy x-rays (5 minutes) or 5 Gy x-rays (10 minutes). Mice which are sham-exposed will be placed in the x-ray machine for similar times. After exposure or sham-exposure they will be returned to their home cages. Mice will be killed following 200 days after their last exposure.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Based on previous experience with our x-irradiation system, we do not expect to see any initial effects in adult mice resulting from the irradiation procedures. In previous experiments, we have not seen any effects in neonatal mice (e.g. rejection by their dam, being found dead) after their return to their home cage and therefore, don't expect to see them in these experiments.

As the experiment progresses and the mice age, some of the mice will bear an increased intestinal tumour burden and may show moderate signs of this disease, including pale feet, ruffled fur, inactivity, lack of appetite, weight loss and may show rectal bleeding or rectal prolapse due to tumours in their colo-rectal region. Mice will be scored using a body-condition scoring system (Ullman-Cullere and Foltz, 1999) and closely monitored for clinical signs of disease and will be killed if they show overt signs of disease.

Some female mice (< 5%) may develop mammary tumours and these will be killed if the tumour is greater than 12 mm diameter (in practice, this is shortly after the tumour has been discovered and before any ulceration has occurred).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Breeding mice.

All C57BL/6 *Apc*^{Min/+} mice will develop intestinal tumours as they age.

Apc^{Min/+} mice used for breeding purposes (about 25%) will be expected to be assigned a moderate severity after 100 days of age as they will have developed intestinal tumours and they may show moderate signs of this disease, including pale feet, ruffled fur,



inactivity, lack of appetite, weight loss and may also show rectal bleeding or rectal prolapse due to tumours in their colo-rectal region.

Apc^{Min/+} mice not required for future breeding are killed and 50% of these will be assigned a mild severity as they will be greater than 50 days old (the earliest age that tumours have been seen to develop). The other 50% of mice will be killed at or just after weaning and they will be assigned a sub- threshold severity as they will show no signs of tumour development.

F1 experimental mice

All F1 C57BL/6 x CBA/Ca *Apc^{Min/+}* mice exposed to x-rays or sham-irradiated will develop intestinal tumours and will be expected to be assigned a moderate severity. Control F1 C57BL/6 x CBA/Ca wild- type mice which are exposed to x-rays will be assigned a mild severity as they will not develop any tumours.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this project is to investigate the effects of ionizing radiation on the gastrointestinal tract. The C57BL/6 *Apc^{Min/+}* mouse model is an established model of radiation-induced intestinal and mammary carcinogenesis and, as such, it provides the best experimental model for this work.

What was your strategy for searching for non-animal alternatives?

Lower organisms such as invertebrates, plants and micro-organisms and intestinal organoids.

Why were they not suitable?

Lower organisms such as invertebrates, plants and micro-organisms are not suitable because they do not have the same gastrointestinal tract structure as mammals.

Intestinal organoids which are multi-cellular intestinal systems developed from intestinal stem cells are proving to be a promising model for the testing of pharmaceutical and food compounds. They can now be cultured in 3-dimensional systems and share many intestinal features including a highly folded epithelium structure consisting of crypts and villi similar to native intestinal epithelium but they lack some of the essential components of the gastrointestinal tract such as the enteric nervous system, vascular systems, lymphatic systems. Some intestinal organoids are being used as models of colo- rectal cancer however, they are unable to reproduce the individual gene losses and the progression of the disease, especially at the early stages and are not representative of the heterogeneous nature of the tumours. The other limitations of intestinal organoids are that



the cells must be embedded in Matrigel™ and before they can be grown in 3-D, which creates organoids that are of differing shapes and sizes and lack the gross anatomical features of the intestine and a few weeks after the seeding of cells to the 3-D scaffold, the model deteriorates which could be due to the lack of nutrients and oxygen starvation. Until the problem of long-term maintenance of the organoids in culture is addressed they will be unable to develop cancer and not able to replace *in vivo* carcinogenesis studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The experimental group sizes have been calculated using power calculations and statistical modelling undertaken from previous experimental results with the aim of using minimum group sizes to produce statistically valid results, aiming for 95% confidence levels.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The numbers of animals used have been minimised on the basis of previous experience with this mouse model and with statistical advice from the in house statisticians and the use of the NC3R's Experimental Design Assistant. Using results from previous irradiation studies on the F1 C57BL/6 x CBA/Ca *Apc^{Min/+}* mice model we have been able to reduce the numbers of mice per group by 50% i.e. from 50 per group to 25 per group.

What other measures apart from good experimental design will you use to minimise numbers?

The numbers of intestinal tumours induced in the model following x-irradiation will be evaluated as the experiment progresses with the aim of minimising the group sizes once statistically valid results have been obtained.

The interpretation of the results will be undertaken with the advice of statisticians and the statistical methods applied will include t-tests, ANOVA, Poisson statistics and regression analysis, as deemed appropriate to the data obtained. Once published in scientific journals all the experimental results will be placed on the STORE database of European Radiobiological Studies (<http://www.storedb.org>.) which will allow future analysis of the results and mean that other researchers will not have to repeat the studies.

The use of animals specifically for molecular studies will be avoided / minimised by long-term archiving of intestinal and mammary tumour tissues.

Refinement



Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice have been chosen for these studies because they are the lowest vertebrate group in which well- characterised models of intestinal cancer have been developed, they are genetically similar to humans and their use maximises the potential use of genetic markers in molecular studies and the potential for interpreting results using genetic databases.

The project will use the C57BL/6 *Apc^{Min/+}* mouse model of intestinal carcinogenesis which is a well- established model of radiation-induced cancer.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We have shown previously that irradiation at immature life stages (embryo and fetus) do show small increases in tumour numbers but giving two doses of x-rays *in utero* may cause their death as immature life stages are known to be more susceptible to irradiation and the mice would need to develop into adults before any effects are seen.

The use of species that are less sentient is not possible as they do not have the same gastrointestinal tract structure as mammals.

The use of terminally anaesthetised animals is also not possible as they will not develop tumours following the irradiation.

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We will only breed sufficient animals to meet out planned experimental needs. All mice will be group housed and maintained to the highest standard for welfare in line with Home Office regulations and approved by our Named Veterinary Surgeon; rodents like to shred, burrow and nibble and hide in dark places, environmental enrichment is included in their cages for them to shred and burrow under, shelters to hide in and tunnels to run through.

Some of the mice will bear an increased intestinal burden and may show moderate signs of this disease. All mice will be closely monitored for clinical signs of disease, and will be humanely killed at the end of the experiment or as soon as they present with overt signs of disease.

Experience gained under the previous Project Licence where rectal bleeding, due to an increase in the number of large intestinal polyps, was observed in experimental groups given 2 and 180 day or 10 and 180 day x-ray exposures has led to a change in the experimental groups in this study. The 180 day exposure groups have been removed from the study and an earlier time-point of 45 days has been introduced. This will also reduce



the maximum life-span of mice in the longer term groups from 380 to 290 days (the average life-span of a F1 C57BL/6 x CBA/Ca *Apc*^{Min/+} mouse is 450 days).

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Compliance with the ASPA (Animals (Scientific Procedures) Act) 1986 regulations - https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/662364/Guidance_on_the_Operation_of_ASPA.pdf

Regular reference to the Animals in Science Regulation Unit Guidance - <https://www.gov.uk/guidance/animal-research-technical-advice>
Regular reference to the ARRIVE Guidelines published by the NC3R's - <https://www.nc3rs.org.uk/arrive-guidelines>

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Attending courses on the 3Rs, use of the NC3Rs website to check for advances in the 3Rs during the course of the project. Literature searches will be undertaken during the study to check on *in vivo* and *in vitro* developments in this field.

Explain the choice of species and the related life stages

We are using a genetically altered mouse model of intestinal carcinogenesis. Adult mice will be used for breeding purposes and neonatal and adult mice will be used for exposures to x-rays in this Project Licence. Studies using aged mice were undertaken in the previous Project Licence; but no effect of a second dose of x-rays at 180 days was observed and so the experiment is being expanded to look for effects of irradiation at younger ages. Based on previous work, It might be expected that the neonatal exposures might give a higher incidence of intestinal tumours when combined with a second exposure which will give much needed information on the effect of age at exposure on tumorigenesis.





134. Regulation of lymphocyte biology by ubiquitin and ubiquitin-like modifiers

Project duration

5 years 0 months

Project purpose

None selected

Key words

lymphocyte, immunity

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

During viral infections, lymphocytes - components of our immune system charged with responding to and eliminating foreign agents - develop from a few small, innocuous cells into many millions of large killing machines able to efficiently detect and kill infected cells. This transformation is accompanied by extensive changes, many of which are poorly understood. We are interested in one of these changes which helps controls how these cells proliferate and function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

These studies may produce several potential bene-fits, most notably in improved basic knowledge about how immune responses are induced, maintained and controlled. Such knowledge may also contribute to our understanding of how these systems some-times go wrong, such as during autoimmune diseases. Furthermore, insights gained from this project are also likely to apply to other bodily systems in health and disease.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?



We use mice, as they are readily genetically modified to allow us to alter specific parts of the immune system so that we can test the function of these components and because mice mimic human physiology very well. We use experimental groups of 5-8 in most cases. We expect to have to breed up to 1000 mice per year for 5 years (the great majority under a 'below threshold' severity limit). Many animals will be used for post-mortem tissue analyses, in which case no in-life experimental interventions will take place.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The majority of mice will be used only for breeding or as a source of tissues & will not be subjected to any interventions. Where mice are used in experiments, these will usually be designed to test how their immune systems deal with different stimuli. These studies will normally involve giving a chemical or micro-organism that promote reactions in the body similar to vaccination. These animals will undergo brief discomfort from the injections to give the substances. There may be possible local inflammation (similar to having an insect bite), and potentially a brief period where animals have a fever / feel "flu-y" or lose a little bit of weight loss because they eat a little less for a few days as a result of immunisation with substances that stimulate immune cells. Most of these effects will be slight (similar to people with a cold) or occasionally may be moderate (more like a person having the flu). Some animals will undergo exposure to radiation, to suppress their immune systems. This is to allow us to monitor how the new immune cells that we give them (taken from the bone marrow of another humanly killed mouse or from a blood sample) move around the body and function. We can't get this information any other way. Animals are kept in a special system of caging after the radiation treatment until the new blood cells have taken over to make sure they have no risk of infection. Once the new cells have moved around the body the animals should have no long-term adverse effects on their welfare. Mice will be humanely killed at the end of any experimental studies.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

We use a type of white blood cell (T-cells) that originally came from human volunteers and are now grown in dishes in the lab to test how different genes work. However, these cells have been changed by living outside the body and do not behave like 'real' T cells from animal or human tissues. In particular, the cells aren't flexible and can't change the way that immune cells do as people and animal age or during infection. The changes that occur during these processes can only be investigated in the lab properly from cells that are taken directly from animals or within living animals.

Reduction

Explain how you will assure the use of minimum numbers of animals.



We will take several approaches to minimise the numbers of animals required. We are careful how we breed animals aiming only to produce enough animals to perform studies and to keep for new breeding pairs. However, even careful breeding practices will produce some animals who can't be used because they don't have the right combination of genes. We will only proceed to using live animals in experiments if preliminary data generated from cells in the lab are strong enough to justify them. We use statistical methods for all our studies that use live animals to check that we are using the right number of animals to make sure that we have the best chance of good results. This means we are using neither too many animals or too few.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The mouse immune system closely replicates that of humans. Although the proteins we are interested in are expressed throughout the body we will generate mice that only lack them in small parts of the immune system, to ensure that potentially harmful effects in other tissues throughout the body are avoided. We use anaesthesia and pain-killers if this is better for the animals. Where we need to use chemicals that change the immune system we will always use the least harm in order to give us the data we need. Similarly, we will always try and give the compounds in a way that is least painful for the animal (so in food or water rather than by injection) whenever we can.





135. Regulation of neural circuits and behaviour in fish by the pituitary and adrenal hormones

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants.

Key words

zebrafish, medaka, stress, behavior, development

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

This programme aims to investigate the effect of early life stress exposure on adult behaviour and physiology using fish. Exposure to stress during early life is a major risk factor in developing psychiatric diseases in adulthood in humans, but the underlying mechanisms are still not fully understood.

One of the major players involved in mediating the stress response are the hormones produced by the Hypothalamo-pituitary-adrenal (HPA) axis such as Glucocorticoid (GC) and Adrenocorticotrophin (ACTH). In this project, we aim to study the effect of altering the level of these two major hormones during development and characterize subsequent behavioural and physiological modifications in fish.

Next, we will identify the brain regions and molecules that are involved in mediating the effects of the GCs and ACTHs. Lastly, we aim to find compounds that can alter the effects produced by early manipulation of GCs and ACTHs. The understanding and identification of molecules involved in GC and ACTH's effect during development may open up new possibilities to tackle psychiatric phenotypes induced by early life stress exposure.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these



could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

This results will provide fundamental knowledge about how key stress hormones alter physiological and behavioural processes. Further, the results obtained will provide candidate molecules involved in mediating the long-lasting and powerful effects of stress hormones in shaping the brain and behaviour. We will further gain mechanistic insight into the function of these molecules. Given the strong conservation of the stress response system throughout vertebrates, the mechanisms that we identify in zebrafish are likely to be also found in mammals including humans. The molecules identified in this project present potential targets for developing therapeutics for stress-induced disorders in humans.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Zebrafish (8100) and Medake (2160) over the course of 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Phenotypes of genetically modified animals produced in this proposal are expected to have no or low adverse welfare effects. However in the unlikely event that breeding of transgenic animals has potentially harmful phenotypes, the offspring will be killed humanely.

The main methods used in this project are behavioural testing of adult and larval stage fish. The behavioural testing sessions involve video recording natural exploratory and social behaviour of animal while animal is allowed to freely swim in a tank. This procedure is expected to produce no harmful phenotypes.

In some procedures we will perform behavioural testings after the animal has been treated with pharmacological compounds. As the treatment duration is short (less than 2 hours), we expect low adverse welfare effects. However, in rare cases, if the experimental animals display compromised movement and visible sign of distress during treatment, the animal will be sacrificed immediately in an humane manner.

In some procedures we combine the behavioural testing with after subjecting an animal to a stressor. Different stressors will be used, but for all of them shortest possible duration of treatment will be applied so that the treatment. The expected level of severity of such a treatment is mild. However, in rare cases, if the experimental animals display compromised movement and visible sign of distress during treatment, the animal will be sacrificed immediately in an humane manner.



Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

The complexity of behavioral regulation by stress cannot be studied using in vitro cell cultures as this requires coordinated interaction of multiple organs. The pituitary and adrenal gland in particular produce multiple hormones in a dynamic manner. We currently do not have possibilities to culture such complex system in tissue culture. Furthermore the stress regulation involving pituitary and adrenal gland is only found in vertebrates. Therefore invertebrate genetic model systems such as fruitfly and worms do not have similar systems. Nevertheless, we will still strive to identify those cases where we can avoid using animals during the experimental design phase. For example, as a large amount of literature on stress biology is available using rodents, we will use this information. Thus, we will only address those questions in zebrafish that have not been already addressed using other species. For some questions, simulation and modelling based on existing data could provide an answer. For those questions, we will seek a close collaboration with mathematicians.

Reduction

Explain how you will assure the use of minimum numbers of animals.

We estimate that we will need to use 8100 zebrafish and 2160 medaka over the course of 5 years. This estimate is based on our own pilot work as well as our own published data. We have extensive experience in studying zebrafish behavior under stress therefore we can accurately estimate the number of animals required for obtaining the results for certain experiments. As more behavioural results become available, we will conduct additional evaluation to see whether the number of animals can be further reduced from those currently proposed. We will also use the latest statistical methods for data analysis to reduce sample size. For our larval behavioral analysis, whenever possible, we will use early stage animals which do not fall under regulation.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Fish represents the most refined animal model for studying the effect of the stress hormones of the Hypothalamo-Pituitary-Adrenal (HPA) axis. The stress regulation of behaviour has been traditionally studied in rodent models especially using mouse. However the HPA axis is a conserved vertebrate feature and can be studied in fish. It cannot be studied in invertebrate genetic model system such as flies or worms.

Optimisation of animal welfare is achieved by different approaches including:

Animals will be exposed for a short duration of stressor treatment.

Pharmacological treatment will use previously published "safe" dose.



The behavioural testing involves non-invasive monitoring of naturalistic behaviour

Animals are monitored continuously during the entire behavioural testing session to look for signs of distress.

At any time during experimental procedures, if animals show clear sign of distress then they will be sacrificed immediately in a humane manner.





136. Regulation of T cell homeostasis and function in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Mice	adult, 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 is the aim of this project?

Our aim is to identify and characterise the mechanisms controlling how particular immune cells called “T cells” are kept in a normal functioning state in both health and in disease conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We do not fully understand how T cells are maintained in an optimally functional state throughout the life course. New T cells are produced continuously throughout life. Immune function declines with age, and it is unclear what mechanisms are defective in aging T cells or the communication pathways within cells that are required for their optimal function. Understanding these pathways is important as they are amenable to manipulation by pharmacological agents.

What outputs do you think you will see at the end of this project?



We will understand how continuous T cell generation contributes to maintaining a full and active immune system, how 'memory' T cells, that can remember specific infections, are made and kept and how memories to new infections are sorted and stored along side existing memories.

Our studies of how communication pathways inside cells control new generation and sustenance of existing T cells, will allow us to better predict how anti-inflammatory therapies influence the function of T cells and what impact this has on host immunity as a whole.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Understanding how T cell-mediated immune responses are generated and maintained has never been more important. Training the immune system to respond to pathogens through vaccination remains a front-line therapy, in the face of the ever growing spectre of anti-microbial drug resistance. Targeted T cell-mediated immunotherapy has finally come of age and is one of the fastest developing areas of cancer treatment. Vaccines and reactivation of anti-tumoral immunity depend on the generation of T cell memory. Therefore, understanding how T cell memory is established and maintained is fundamental for development of such therapies.

How will you maximise the outputs of your work?

We aim to publish all novel, robust research findings consequent from the project, whether results represent new findings and insights or simply support a null hypothesis. This will be achieved by publication but also storing manuscripts with pre-prin servers such as BioRxiv to maximise speed of dissemination of new data.

We also collaborate extensively with mathematical modellers who perform sophisticated modelling meta analysis of data to extract the maximum information possible.

Species and numbers of animals expected to be used

- Mice: 12000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, we study the function of immune cells following either genetic or pharmaceutical intervention. Transplantation of immune cells is also a key method to be able to follow the behaviour of a defined cohort of cells in the context of a normal immune system. As such, mice will typically be subject to drug conditioning, to allow injection of cells to become settled and established in a new host. Mice may then be subject to blood sampling to allow longitudinal analyses or killed to allow more detailed analysis of immune cell function. A



single procedure would therefore typically involve 3-6 separate steps and monitoring continue for as few as a few days or as long as several months.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

For the majority of experiments, analysing immune function following an intervention of some kind, mouse health and behaviour is not expected to be altered as a consequence. Infection of mice with specific infectious agents such as influenza virus, may result in transient weight loss, from which most mice recover. The condition of mice will be carefully monitored and weight loss not permitted to progress beyond defined limits, instead killing the mouse before severity limits are breached.

Establishment of tumours is not expected to impair normal function or health. Nevertheless, tumour size will be strictly monitored to ensure tumour size does not exceed defined limits and result in impairment to physical function of mice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

For 70% of experiments, we expect only a mild severity. For mice either subject to live pathogen infection or tumour engraftment, we expect severities to reach moderate levels.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 the complex physical and temporal arrangement of the immune system, whole body model systems are required to unravel functions under homeostatic conditions as well in inflammatory scenarios. While it is possible to generate information regarding regulation of some aspects of the immune cell behaviour in cell culture it is not possible to mimic homeostatic or immune responses to infection in vitro.

What was your strategy for searching for non-animal alternatives?

We considered using in vitro cellular immunology assay systems and immortalised cell lines.

Why were they not suitable?

In vitro systems, while useful, do not fully replicate the complexity of immune interactions or disease pathogenesis in vivo and it is essential to use appropriate and robust animal models to dissect these processes. Immortalised cell lines can be useful tools for testing



broad principles or signalling pathways, the way in which signalling pathways are configured varies considerable between different tissues and indeed, the same cells at different stages of differentiation. Therefore, studying primary cells in vivo is critical to understand the context specific functions we proposes to investigate here. Furthermore to develop therapeutic approaches with potential to alleviate human disease it is necessary to establish parameters influencing efficacy in an animal model.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

It is difficult to predict the full course of experiments that will be undertaken in the course of pursuing a project over a five year period. However, individual experimental design are subject to power calculations to estimate the number of mice required for a single trial and then the number of trials required to provide statistically robust evidence of any differences.

For objectives 1 and 2

Approximately 30 GA lines are required for these objectives, although other novel strain combinations may be required as dictated by experimental findings during the project. Generating novel strain combinations to test gene functions genetically is a key component of the proposal. As such, generating and maintaining the 30 or more lines required for the proposal will require a breeding colony of approximately 100 cage capacity using approximately 600 mice/year (assuming an average 4 month breeding life per pair).

To establish statistically robust results for monitoring immune responses by T cells in vivo, or comparing mouse strain phenotype will typically require 3-5 independent replicates using 10-16 mice per replicate and a total of between 30-48 mice for a given strain, including littermate controls. We estimate that a single experiment a month for each research associate (of which there are 3), using ~20 host mice, will require ~750 mice /year. ~2/3 of these experiments will be under protocol 2 and ~1/3 under protocol 3. Therefore we require approximately 2500 and 1250 procedures for protocols 2 and 3 respectively over the five years of the project.

For objective 3

Generating and maintaining the 10 lines and congenics required for this objective will require a breeding colony of approximately 50 cage capacity using minimally 300 mice/year (assuming an average 4 month breeding life per successful pair, but ~10% breedings fail).

To establish statistically robust results for monitoring T cell behaviour/phenotype in vivo, or comparing mouse treatment and control groups will typically require 3-5 independent trials using 10 mice per trial and a total of between 30-50 mice for a given condition, including littermate controls. We estimate that two experiments per researcher (of which there are 3) a month, using 20 host mice, will require ~750 mice/year. Approximately half of these



experiments will be under protocol 2 and remaining experiments split between protocol 3 and 4. Therefore we require approximately 1900 procedures for protocols 2 and ~950 procedures for each protocols 3 and 4 over the five years.

Summary

Protocol 1 : ~5000.

Protocol 2 : ~4400

Protocol 3 : ~2300

Protocol 4 : ~1000

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We have taken advice from statistical collaborators to take an experimental approach that aims to maximise the detection of experimental effects in a given trial, and then to test for reproducibility of experimental findings between trials. For mathematical modelling experiments, we use just as many mice as are required to allow robust estimation of model parameters. These are typically kinetic experiments and concentrating mouse sampling over the periods of greatest dynamics is critical for this. Conversely, fewer mice need be sampled in less dynamic periods. Such uneven distribution of sampling results in most efficient use of mice. Model fitting can also identify areas of uncertainty that would benefit from improved sampling frequency. Such iteration between experiment and modelling is a central principle in avoiding oversampling and therefore redundant use of mice.

What other measures apart from good experimental design will you use to minimise numbers?

Whereever possible, we use both male and female mice.

Breeding strategies for GM REDACTED ensure that, where possible, control groups can be taken from littermates or that control and experimental GM lines are derived from common parentage to ensure background genetic composition is equivalent.

Use of multiparameter single cell analysis methods permits several experiments to be performed and data collected from individual mice - studying more than one immune cell type from the same individual for instance.

Mathematical modelling is an integral methodology of our work. This informs optimal experimental design but also substitutes for empirical experimentation. Models make predictions that can be validated by much smaller experiments rather than large empirical experiments that might arrive at similar conclusions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.



Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

In order to achieve our goals we propose to use mouse models to study the development and function of the immune system for several reasons. Mouse transgenic and knockout techniques are well- established and their haemopoetic system has been intensively studied and bears extensive similarities to that of humans. There exists a vast array of reagents facilitating the study of the immune system in contrast to the situation in other documented species.

Our procedures are of mild/moderate severity and have clearly defined endpoints. However, it is not possible to fully predict the nature or severity of any potential defect and for all types of mice there will be careful monitoring for possible adverse side effects. Careful monitoring of strain characteristics will be employed and the information collated on databases to monitor whether phenotypes exceed their usual characteristics. Animals exhibiting any unexpected harmful phenotypes will be killed using a Schedule 1 method, or in the case of individual animals of particular scientific interest, advice will be sought from the local Home Office Inspector.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

To our knowledge no other species of lesser sentience can fulfil the requirements of this programme to the same extent as the laboratory mouse.

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We have gained a detailed understanding of the genetic and chimeric mouse systems employed in this project. We have specifically developed the use of the conditioning drug busulfan to allow generation of bone marrow chimeric mice. This represents a substantial refinement of the traditional conditioning methods of lethal irradiation of mice, which have numerous well documented welfare costs to mice. Drug treatment is non-lethal and, at the doses employed experimentally, very low toxicity.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

With assistance of our local biological services staff, we monitor the 3Rs literature and newsletters, as well as scientific literature for new opportunities for refinement. For administration of substances and aseptic surgical techniques, we have established best practices informed by guidelines published by the Laboratory Animal Science Association.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We remain alert to technological advances that permit refined use of mice or replacement with in vitro model systems, as well as monitoring the 3Rs literature and newsletters.

Explain the choice of species and the related life stages



The aim of our research is to better understand how the immune system is maintained in a healthy functioning state throughout life. Because of the complex physical arrangement of the immune system, whole body model systems are required to unravel functions under homeostatic conditions as well in disease scenarios. While it is possible to generate information regarding regulation of some aspects of the immune cell behaviour in cell culture it is not possible to mimic homeostatic or immune responses to infection in vitro. The mouse represents a relevant and tractable system to study the immune system. Mice are readily amenable to genetic manipulation, which is a key tool exploited in this project. The immune system of the mouse, next to that of human, is the best characterised immune system of any organism, and for which there are the greatest number of tools and reagents available to study. The natural life time of the mouse, at around 1-2 years, makes possible life long studies of immune function.





137. Repair, replacement and regeneration of cartilage and 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
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Sheep	juvenile, adult, aged
Rats	juvenile, adult, aged
Rabbits	juvenile, adult, aged
Pigs	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 is the aim of this project?

Evaluate novel therapies or technologies to facilitate the repair, regeneration or replacement of cartilage or 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?

Cartilage has important functions for joint lubrication, ease of movement and shock absorbing. Damaged cartilage can be extremely painful and can significantly impact quality of life. Cartilage has limited repair capacity: when cartilage is damaged and repairs without



help from medicines or surgery, it is often of lower quality than the original tissue, with reduced shock absorbing properties. In joints, severe damage to cartilage can be accompanied by damage to the bone surface beneath. Repairing damage to bone is applicable to multiple clinical settings, from traumatic head injuries, to craniofacial and maxillofacial reconstruction, to dental surgery.

Repair or regeneration of cartilage and bone can be affected by multiple mechanisms and factors, such as the formation of new bone cells, tissue and new blood vessels, mechanical stimulation or weight bearing. The efficacy of a technology to assist cartilage or bone tissue regeneration, or replace damaged tissue, must be evaluated in the context of these interacting factors as much as possible, *in vivo*.

Surgery, cell therapy (including stem cell and cartilage cells), or physical implants (e.g. plates, screws or artificial joints) are currently the major strategies to repair or replace cartilage and bone. The optimum strategy must be stratified to the patient's individual needs.

The work conducted in this project can answer lots of questions about new strategies and technologies to repair, regenerate or replace bone or cartilage, for example: how well can different materials replace or repair damaged tissue, how should they be attached, are they compatible with the existing intact tissue; ways to administer stem cells, ways to improve stem cell therapy (for example how many stem cells to administer and when and for how long)? The models and protocols described in this project will be applicable to multiple clinical fields, from osteoarthritis, to sports injuries, to head trauma, craniofacial or maxillofacial trauma, to dentistry.

What outputs do you think you will see at the end of this project?

Data will support product development, product registration, patents, and ultimately lead to new products.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

For research Sponsor companies, data can assist elimination of inadequate candidates prior to further costly development, and further animal research.

In the long-term these studies will benefit patients with various musculoskeletal diseases or conditions:

the impact of this work is applicable in multiple treatment fields.

How will you maximise the outputs of your work?

Whilst publication of results would not be discouraged, it is expected that in most cases publication or dissemination will not be possible as the research will be conducted as a commercial service to companies.

Internally, the organisation will review processes and procedures after the completion of experiments, and use that experience to inform and develop future studies.

Species and numbers of animals expected to be used



- Pigs: 150
- Sheep: 150
- Rats: 150 Rabbits: 150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will undergo invasive surgery to create injuries in bone or cartilage. These injuries may include:

damage to the cartilage and/or bone in one knee joint of a sheep, or complete removal of a piece of cartilage (the meniscus) in one knee joint of a sheep;

removing a piece of bone from the top of the skull of a rat, or removing multiple pieces of bone from the top of the skull of a rabbit, pig or sheep.

These injuries will then be treated with new drugs or materials. In some cases, the injuries may be left untreated as a comparison to the new treatment under investigation.

The injuries will be created under general anaesthesia which could last up to 3 hours for larger animals, depending on the defect or treatment required. The recovery from anaesthesia can be around 1-2 hours with prolonged anaesthesia like this, during which animals will be uncomfortable and disorientated. Pain and discomfort is expected during the subsequent recovery period (days – weeks), which we will manage with pain relief, and antibiotics will be provided if required. These will be administered with food, as injections, or as dressings.

If a drug or cellular treatment is being investigated, these may need to be administered on numerous occasions during the experiment, for example with food, as injections, or dressings. We may also need to take multiple blood samples from the animal during the experiment. The animals will need to be restrained for these procedures, and occasionally it may be useful to anaesthetise them. To prepare for this the animals are handled regularly to get them used to the staff and reduce any distress caused by restraining them as much as possible.

The animal may need to be anaesthetised on other occasions after the surgery to take CT images of the injury, for example to see how well the injured tissue is repairing, or if the test material is still in place. These sessions of anaesthesia will be quicker than the initial surgery, but the animal will still need to take medication and need some time to recover afterwards.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.



Pain, discomfort, lameness or reluctance to bear weight on operated legs is expected during the recovery period after surgery, and these signs may be accompanied by some weight loss. We would expect these signs over a 1-2 week period (with improvement over this time frame) particularly for more invasive surgeries on larger animals, such as the joint surgeries on sheep.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

All animals are likely to experience moderate severity effects as a result of the protocols described, although this is expected to be short-lived.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cartilage and bone are complex tissues which interact with surrounding matrices and tissues and whose growth and maintenance is affected by the mechanical stimulation of joint motion and weight bearing. These conditions are almost impossible to replicate in *in vitro* or *ex vivo* tissue when looking at functional healing or remodelling especially over longer time frames (i.e. months).

What was your strategy for searching for non-animal alternatives?

Non-animal alternatives have not been considered for this project, as the aim is to evaluate products and treatments in a replication of the clinical setting, including multiple factors of movement, weight bearing, the environment within the joint and blood supply for example.

It is expected that research conducted under this project licence will be based on supporting *in vitro* and *in vivo* evidence.

Why were they not suitable?

Non-animal models, for instance mechanical models or cell cultures, could replicate some of the factors involved in the healing process, but would not provide good information on integration, longevity or wear of implants for example, or replicate multiple important factors simultaneously such as the environment within the joint or surrounding tissue, weight bearing or blood supply.

Reduction



Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated animal numbers are based on examples of likely study designs and timespans, for instance:

up to two large animal (pigs or sheep) studies a year, using a maximum of 15 animals per study (150 animals per 5 years),

one GLP study in smaller animals (e.g. rats) per year, requiring 30 animals (e.g. one group of 30 or one group each of 15 males and females, according to OECD guidelines).

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

For experiments using the joint, one leg will be operated on, leaving the opposite leg as a control. For skull defect experiments, multiple injuries can be created in larger animals (pigs and sheep), so control conditions can be measured alongside treated conditions. This helps to eliminate the need for separate control groups in most studies.

We encourage the use of non-invasive imaging and measurements (for example CT scans or observing weight bearing) to eliminate the need for including extra animals to be killed at earlier timepoints.

Pilot studies with 1-2 animals can be conducted, for example for significant changes to products or procedures, before commencing larger studies - where possible, pilot study data will be included in the main study.

We frequently recommend cadaver studies to Sponsors, and use cadavers for model development and training opportunities.

What other measures apart from good experimental design will you use to minimise numbers?

Pilot studies with 1-2 animals can be conducted, for example for significant changes to products or procedures.

In skull injury models in larger animals, we can replicate treatment conditions within each animal as well as between animals, which increases the number of measurements we can take as well as improving the reliability of these measurements.

We encourage the use of non-invasive imaging and observations (for example CT scans or observing weight bearing) which can give us extra functional information during the study, rather than just looking at tissues at the end of the study.

We routinely harvest tissues and organs from experimental animals for research and training purposes.



Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Sheep models are often used for research into osteoarthritis, joint damage and repair. Sheep joints are a close representation of human joints mechanically and the thickness of cartilage is more similar to humans than other animals. For cartilage repair studies, just one leg will be operated on and medication will be provided to reduce pain and minimise the risk of infection, so movement and weight bearing will be disrupted as little as possible, other than the discomfort following the surgery and injury itself.

Calvarial (skull) defect models are often used for research into bone regeneration. These are not load bearing and do not interfere with mobility. In smaller animals, these can provide basic and mechanistic information in shorter timespans than larger animals, for instance studies. In larger animals, information can be gathered on multiple treatments in larger injuries and over a longer timespan.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

For protocols 1 and 2 it is not possible to use smaller species as the differences in bone size (and therefore the size of implant used and the load applied to it) and the growth rates of these animals compared to humans makes the information we gather less accurate and less useful.

More skeletally mature animals are more useful for these purposes. For bone regeneration studies (protocol 3) it is possible to use less sentient animals (mice, rats) in some cases, e.g. for basic research or mechanistic studies. However it will sometimes be necessary to investigate bone regeneration in larger defects or over larger time periods, and less sentient animals would not be suitable for these studies.

Zebrafish and amphibians are an emerging model for orthopaedic research, but fish bone structure is diverse and there are larger cellular and structural differences between fish and human bones compared to other mammals, and the regeneration of amphibian bones in response to injury can differ widely from that of humans. For functional assessments of injury repair with novel treatments in a clinically relevant setting, the presence and structure of the long and flat bones along with the requirement for particular articulated joint movement and weight bearing means mammals are a more appropriate model for these projects than fish and amphibians.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?



We routinely provide post-operative pain relief and antibiotics, and are prepared to extend the provision of these where necessary. All animals are closely monitored immediately after surgery, and then observed closely in the days and weeks after surgery, for instance their mood, grooming, movement, weight, eating and drinking, how the wounds look.

All the animals arrive to the facility at least two weeks before studies start. We try to receive larger animals for larger studies even earlier (e.g. four weeks) so that they can get used to the new surroundings and staff. During this time staff can start to train the animals if this will be needed for the experiment, for instance to stand still for blood sampling. Smaller animals (rats and rabbits) will also be handled frequently before the study to get them used to staff and being handled.

All animals are provided enrichment whilst in the facility, such as chew sticks or treats and raised tunnels for rats; raised steps, a hiding place and dietary enrichment for rabbits; chains, balls and bars for pigs to chew; chews and room to explore for sheep.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Our facility is GLP-certified and experiments can be performed to GLP-certified standards where required; non-GLP studies are performed to the same standard operating procedures.

The PREPARE guidelines cover many topics that are usually discussed between the research team and Sponsors whilst developing protocols, and an adaptive approach to these will be useful for planning specific studies and continuing the project.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Our research staff actively seek opportunities for training and continuing professional development. We circulate the NC3Rs newsletters and take part in IAT and LASA activities. We communicate openly about animal welfare and suggestions and ideas for improvements are welcomed from all staff members.

Explain the choice of species and the related life stages

Using bone injuries in rats and rabbits is a useful way to investigate the properties of new treatments in the short-term; the injuries created are much smaller than in larger animals and the healing will be quicker. This is an efficient way to investigate or compare new treatments quite quickly. Juvenile or adult animals would be used as their general growth rate should remain constant during the course of experiments.

Larger animals (pigs and sheep) are useful to evaluate products in the final stages of pre-clinical development: scaffold and implants of a similar size to those which would be used in humans can be applied; larger defects can be studied; and repair can be monitored over a longer time period (as could be expected in the human clinical setting). The size and weight of sheep, and the thickness of their cartilage, are more similar to humans than other laboratory animals. Juvenile or adult pigs and sheep would be used as they will be more skeletally mature and their general growth rate of new tissue should remain constant during the course of experiments.





138. Respiratory system models

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

No answer provided

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 is the aim of this project?

To investigate and target mechanisms involved in diseases of the respiratory system, and where the respiratory system provides a relevant model, to investigate mechanisms involved in other immunological and inflammatory clinical 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?

Despite progress in the discovery and development of medicines, there is still a significant unmet need in the treatment and prevention of respiratory diseases (that affect lungs and airways), and diseases that involve inappropriate function of the immune system (immunological diseases and disorders). For example, within the EU, it was reported in 2015 that diseases of the respiratory system accounted for 8.5% of all deaths although in the UK specifically this stood at 14.1%.



Other diseases with high personal, social and economic costs (e.g. allergies and inflammatory diseases) have underlying immunological causes. The National Institutes of Health (NIH) estimated in 2006 that up to 23.5 million Americans suffer from autoimmune disease that are typically disorders in which the immune system attacks the body in error rather than concentrating on attacking non-self ('foreign') cells or targets. This is the third most common category of disease in the United States after cancer and cardiovascular disease, and reviews show that the prevalence of these types of diseases is rising (2019 information). The US National Institutes of Allergy and Infectious Diseases (NIAID) estimated in 2011 the cost of treating autoimmune disease in the US as greater than \$100 billion annually.

The respiratory system with immune system involvement is a relevant system to investigate how respiratory and immunological disorders occur and develop. It can be used to investigate potential new targets and biological pathways common to some of these diseases, and to check that new treatment approaches work. Through examining information obtained from patients and determining how immune biology and disease are linked by genes, it is anticipated that new, specific and effective ways of treating diseases will be identified. Where targets are thought to be involved in development of respiratory disease, the use of animal studies designed to assess respiratory system changes will be essential to identifying and assessing possible new treatments.

What outputs do you think you will see at the end of this project?

This licence supports a wide range of methods to enable investigations of inflammatory and immunological systems that underlie many types of diseases, including those of the respiratory system. The respiratory system will also be used as a relevant model environment to provide information on common mechanisms that underlie different systems and disease indications (to enable subsequent expansion of research into a wider range of disease indications when mechanisms in common exist).

Studies will generate essential information to support scientific programmes aimed at identifying and understanding new ways of treating diseases of the respiratory system, or to investigate immunological mechanisms. Experiments will be carried out to establish if animal models focused on parts of the respiratory system allow assessment of immune or inflammatory processes, or processes involved in other diseases which share mechanisms.

We will generate data on the effectiveness of potential new medicines in comparison to other treatments, and determine how the medicine might distribute throughout the body. This will aid selection of potential new drugs for the treatment of both respiratory diseases, and for other immunological and inflammatory conditions. The data will contribute to medicine development programmes to address unmet medical need in a range of disease types, to benefit patient health. It is also anticipated that work carried out under this licence will be included in scientific publications to enhance scientific understanding in this field.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The information generated from this work will contribute to medicine development programmes to address unmet medical need in a range of disease areas.



It will be an early step in a long process to confirm the relevance of targets that are involved in disease or to confirm how well a potential new medicine works, and will be used to support later clinical trials when new medicine effectiveness is tested in humans. Patients will benefit over relatively long timescales because medicine development can take many years.

It is intended that there will also be shorter timescale benefits to society derived from work carried out under this licence through contribution to this field of science. It is the intention that information derived from our work will be published in the scientific literature.

How will you maximise the outputs of your work?

Work is expected to be included in scientific publications as part of the process of investigating and validating new disease targets and new treatments for disease. Additionally where work is considered to be 'pre-competitive' (e.g. method development or model validation work), whereby it does not contain information that is subject to intellectual property constraints, it will be considered for publication.

This continues an established approach, as demonstrated by previously published work as part of consortia such as the EU Innovative Medicines Initiative that ran between 2010-2015. Additionally, REDACTED also supports the view that publication of unsuccessful approaches ('negative data') is a valuable scientific output from properly conducted research, and this type of data would not be excluded from a publication strategy.

Information generated using this licence is stored in a searchable backed up company database, so that it is always accessible to other internal company researchers. Therefore, data will be recoverable in the future, even after likely project and personnel changes, and the information will be a valuable future resource to reduce the need to repeat and re-establish competency in a field of research.

Species and numbers of animals expected to be used

- Mice: 9000
- Rats: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

In most cases, an immune response will be initiated in an animal through exposure to an agent such a virus (e.g. influenza) or bacteria that engages with the innate immune system (the part of the immune system that produces a quick and general response to an environmental challenge) or will use an agent such as an allergen that will trigger an adaptive immune response (a very specific response that takes time to develop but which then will exist for a very long time). Agent exposure could be via injection using a syringe or direct to airways of the respiratory system by dosing liquids through the nose or direct to the windpipe whilst an animal is briefly and lightly anaesthetised. Alternatively, animals



could be exposed to an irritant or chemical vapour within a chamber over minutes or hours to start an inflammatory process.

Many animals will have samples or measurements taken during experiments (e.g. blood samples taken from veins), or they may be anaesthetised so that imaging of organs can be carried out to determine disease progression, and to determine how effective an ongoing treatment may be. The types of imaging methods would include use of X-rays or magnetic resonance imaging (MRI), similar to 'scans' that humans might experience in a hospital visit.

Many animals will receive potential new medicines, either during or after immune responses are established or changes to the respiratory system have occurred. Such compounds could be dosed via a variety of administration routes (e.g. oral, intravenous or via inhalation) on single or multiple occasions, over days or weeks. Some animals could undergo brief surgery to implant dosing devices that slowly release the medicines over hours or days.

All animals will be humanely killed at the end of each experiment to enable collection of samples that cannot be ethically collected from live animals without unacceptably compromising their welfare (e.g. taking large blood volumes or taking whole organs for study, such as lungs).

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

In the majority of cases animals will not show specific signs of distress or abnormal behaviour following initiation of immune responses, although body weight loss may be noted (e.g. 5 - 10% reduction in weight compared to before treatment, lasting several days).

Following exposure of mice to some strains of influenza more pronounced weight loss is noted (up to 20% loss of bodyweight) peaking about a week after exposure, although mice typically recover weight within a further few days.

Sometimes additional adverse signs can be noted after different types of treatments, (e.g. injections of agents that activate the immune system). These activities could result in reduced mobility (usually for up to several hours) and raised or stiffened fur for periods of up to several days before signs disappear.

Mice exposed to bacterial airway infection (of the strains characterised for use within this institution) do not normally demonstrate adverse effects over the timescales of most experiments, but animals will always be monitored for weight changes and appearance of any clinical signs.

Rats that are exposed to aerosols or inhaled dry powder formulations of potential new drugs usually show only mild 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 species)?



Experience during work under previous licences indicates that at least 60% of mice are expected to show no more than mild effects following treatment. Up to 40% of mice will likely, demonstrate a cumulative moderate severity experience, or at least a period of moderate severity at some point during experiments.

The majority of rats (greater than 75%) will likely experience cumulative mild severity, and up to 25% of rats will experience moderate severity.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Living animals are able to function because of complex coordinated systems that are made up of many different types of cells in separate organs. Some parts of these systems can be reproduced in isolated artificial plastic (*in vitro*) experiments, but the integrated interactions between cells and the effects of those interactions on wider processes and mechanisms that are, for example, found in the respiratory system are not easily and completely replicated outside a living animal. The inflammation and immunological systems that help to keep the body healthy are very complex, highly coordinated, dynamic communication networks that can respond quickly to injury or infection. They consist of sequences of events that are common and conserved in mammalian species, and hence results from experiments carried out in rodents can be compared and translated to the human setting. These responses involve many different cell types (such as white blood cells) as well as signalling molecules that are produced by those cells or the layers of cells (known as epithelium) that line the airways of the respiratory system. Responses are coordinated to identify disease causing organisms (e.g. a virus or other pathogen) or damaged tissue, and act to alert and recruit other cells and signalling molecules or activate processes that can lead to structural changes in organs.

Additionally, investigations into disease states that are the result of altered or mis-functioning mechanisms, or investigations into highly organised body systems require the use of mature functioning tissue that cannot currently be fully replicated, grown or kept alive outside animals (*ex vivo*). The mucociliary clearance system (a good example of such a coordinated mechanism) that is composed of mucus layers and beating cilia within the airways and lungs acts to clear foreign bodies from the respiratory airways. Whilst a wide range of information from isolated cell systems is generated as part of the investigatory process to increase our understanding of basic mechanisms and how potential treatments target or affect many functions, understanding the integrated response in a whole animal with a physiology that is common with humans is vital to guide potential medicine progression to human clinical trials.

What was your strategy for searching for non-animal alternatives?

Models using types of isolated human and animal cells have been developed and widely published in the scientific literature, and are used as part of development of new



medicines. The vast majority of these are models that use flat cultures of cells growing on plastic that are able to address focused questions to a certain level of complexity with a limited number of cellular interactions.

The continuing progress with development of in vitro three-dimensional modelling systems such as 'lung on a chip' and epithelial organoids have started to address some of these challenges with cellular interactions, but are not yet at the levels of complexity required to mirror the clinical disease situation.

Why were they not suitable?

Despite much progress since being first published in 2010, the three dimensional (3D) lung-on-a-chip systems and model derivatives do not yet provide the full level of complexity and integration available within a living animal, or provide the inter-connected body-wide systems where cells and organs continuously communicate. Lung on a chip development has also concentrated on the gaseous exchange parts of the lung rather than conducting airways which play an important part in many aspects of respiratory disease. Additionally, systems using cells are not yet adequately able to model potential drug distribution and elimination from the body which results from a compound passing through organs such as liver and kidneys.

Non-animal systems such as organoid cultures which are 3D cellular structures held within a matrix gel, are not yet characterised to a stage where we can have confidence in the clinical relevance of data generated from using them. Tissue slice models are also used as in vitro tests. These experiments involve taking a tissue such as lung and preparing many small pieces that can then be used for individual tests, but in most cases those models, and also cell based models, lack physiological relevance due to the test system not experiencing the flows, physiological oxygen concentrations and pressures that exist within the blood vessels and airways of living animal systems. As such, those technologies are not yet able to model closely enough the complex environments needed for a system to be used to translate results to a complicated disease-like situation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimates of animal numbers that are likely to be used over a five year period are based on the experience of related work and projects previously supported under licence authority within REDACTED. There is an anticipated steady rate of projects likely to require information that cannot be gained from non-animal alternatives. Also considered in these estimates is the likelihood of changes to research priorities as a result of continuing scientific advancements in many areas of science. Because we are familiar with the types of models that are likely to be used and know the resources that we have available we can estimate the number and type of studies that will likely be required over the life cycle of a licence.



We use accepted statistical principles based on the main readouts from each model together with knowledge of the variability those readouts to inform on animal numbers required per type of study to produce statistically useful information.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

All experimental work is planned with input of statisticians to ensure that experiments provide high quality data without using an unjustified number of animals. For example, the size of experimental treatment groups will either be based on existing data, or small trial (pilot) studies will be carried out to generate guidance information that will show the variation of the biological system and readouts being assessed. Understanding variation and what constitutes a meaningful biological response to a treatment allows a statistician to calculate treatment group sizes that can be used to ensure that statistically meaningful comparisons can be made between treatment and control groups. These design principles aim to reduce the possibility of experiments not generating decision making data that could lead to the need to repeat work and hence use more animals.

All experiments undergo review from a panel of multi-disciplinary and independent specialists in planning stages. Experience has demonstrated that independent peers bring a range of viewpoints and specialisms to the design of animal studies, resulting in challenge to convention.

Where the effects of potential new medicines or control treatments are tested, we will often include an assessment of the blood or tissue levels of that substance from the same animals that are being used to look at effects of treatment. This allows direct comparison of treatment effects and compound levels and reduces the number of animals required overall. Animals will be randomly assigned to experimental groups using a random number generation system. This will reduce bias in a design that could compromise the value of generated data and potentially lead to more animals having to be used if work were to be repeated. Additionally, for subjective readouts that require a person to make a judgement, bias in data interpretation will be avoided by using experimental blinding. Thus, those involved in making those assessments will not be influenced in the interpretation of readouts by knowing which treatment had been given to animals.

These robust study design measures will maximise the likelihood of generating non-biased experimental results, and limit the number of animals needed to generate good quality decision making data.

What other measures apart from good experimental design will you use to minimise numbers?

To reduce variation in biological readouts we will ensure that our facilities provide a constant optimal environment suitable for the species, and we will control the number of personnel involved in making any subjective experimental measures. By controlling variation in readouts, we aim to ensure that animal group sizes are the smallest possible to achieve the scientific objectives of the study.

For some model systems, rather than collect only terminal measurements we will employ in-life imaging such as MRI (or serial sampling) where we can take images or samples repeatedly from the same animals at different time points to monitor development of pathology or changes resulting from treatment. This approach will reduce the total numbers of animals required to gather decision making data because each animal will act



as its own control, thus reducing readout variability and improving quality of data. Statistical information generated from analysing such data is likely to be more robust compared to data derived from single measurements from many different animals. The same principles apply to taking serial samples (e.g. blood samples) from fewer animals, provided that animal welfare or sample quality are not compromised.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Different models of short and long term immune system sensitisation and challenge, targeting distinct aspects of the immune system will be used to investigate the involvement of those processes in human disease. Some models will use chemicals derived from bacteria to mimic the effects of bacterial infection, or use chemicals that trigger the immune system to act as if a virus had started an infection. Sometimes proteins will be given to animals along with chemicals that boost the immune response (acting as an adjuvant) that together cause the immune system to react. The same protein put later into the airways of the respiratory system would then trigger a localised immunological allergic reaction that can be used to test treatments targeted at aspects of the induced immunological pathway in that system. The project will also use extract of house dust mite which is a commonly detected allergen associated with respiratory diseases such as asthma. Some models will include viral or bacterial microorganism infection with or without additional challenge. These microorganisms will be tested in mice because it's known that this species is susceptible to both respiratory infection and allergen- induced respiratory system inflammation.

The animal models used for this programme of work have been chosen and, where necessary, will be developed further to represent the least severe and most effective and robust ways of measuring immunology and inflammatory mechanism changes over both short and longer duration experiments. The durations of experiments will be suited to the mechanism under investigation, and in the majority of cases pain, suffering or distress will be brief and symptoms will be transient. Any airway inflammation or infection changes in these experiments is usually mild and would normally not cause any obvious signs of distress. Body weight loss is known to be a sensitive measure of the severity of infection. Changes in weight will be monitored to assess the models, and also limit the possibility that an animal might develop adverse effects that are more severe than are needed to reach an answer to a scientific question. Each approach described above models distinct aspects of human diseases or processes involved in multiple diseases and are carried out for only as long as required for the relevant conditions to develop and potential new treatments to be assessed.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?



Rats and Mice are the lowest orders of animals that have immune responses similar to humans and, additionally, allow us to take blood samples as well as monitor how the immune system is functioning during treatments. This means that we can correlate the level of potential medicines in the bloodstream with the effects on the immune system in the same animal at the same time.

To undertake the work proposed in this licence the animals must have reached an age where they have functioning immune systems, and this precludes use of animals of less than 6 weeks of age (but preferably of at least 8 weeks of age). By this age immune system responses have matured to a sufficient extent to include working innate and adaptive systems (that can fight a broad range of potential attacks against the body), and have immunological memory (the developed ability to quickly recognise something that the body has previously been exposed to, such as an allergen). Most immune and respiratory system model assays last for several days and it is therefore impractical and unethical to keep animals anaesthetised for the duration of the sample collection periods. Even the shortest investigations of adaptive immune responses require greater than 6 hours for responses to develop, and therefore, it would not be possible to carry out the majority of procedures under terminal anaesthesia.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals on studies will be monitored daily, and any adverse clinical signs that could be treatment related will be recorded and scored by reference to a specific scoring system for each type of experiment, therefore allowing monitoring of potential trends and modification of treatments if necessary.

We will continue to investigate the use of non-terminal imaging techniques that allow repeat measurement of disease relevant parameters (e.g. changes to sizes of organs and vessel walls, flow rates of blood, amount of swelling, fluid build-up) within same subjects during the development and treatment of pathologies, e.g. pulmonary hypertension. These within-subject measurements over time carry great statistical power because inter-animal variability is reduced, and thus smaller treatment groups are needed to demonstrate statistical differences between treatments. When using imaging methods we may also change the type of diet to increase imaging quality by reducing background environmental fluorescence. The nutritionally complete chlorophyll free diet used in place of standard rodent diet will allow improved imaging data to be collected without affecting animal welfare. The licence holder will also maintain familiarity with this field of science to make sure that relevant advances in the scientific area can be incorporated in work.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The published principles and philosophies behind the PREPARE (2018) and original ARRIVE (2010) guidelines have been incorporated into the sponsoring company's internal project planning standards of care and standard operating procedures. All work carried out under authority of this licence will undergo assessment of the study design during planning stages as part of a peer review process that is based on those guidelines, and will include statistical consultation. Facilities and processes are audited by independent bodies such as AAALAC International which has published guidelines and procedures to ensure work is carried out to high ethical and humane standards. The following published documents



will advise on experimental design, animal welfare and husbandry during the life cycle of this licence:

Kilkenny C et al (2010). Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. PLoS Biol 8(6).

Smith A et al (2018). PREPARE: guidelines for planning animal research and testing. Lab Anim; 52(2):135-141.

Percie du Sert N et al. The ARRIVE guidelines 2019: updated guidelines for reporting animal research. BioRxiv. 2019: 703181.

NC3R's - Responsibility in the use of animals in bioscience research: expectations of the major research council and charitable funding bodies (2019).

Guidance on the operation of the Animals (Scientific Procedures) Act 1986. (Home Office 2014).

LASA - Guiding principles on good practice for animal welfare and ethical review bodies. (2015)

Prescott MJ, Lidster K. Improving the quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156, (2017).

Review of harm-benefit analysis in the use of animals in research. Report of the Animals in Science Committee Harm-Benefit Analysis Sub-Group chaired by Professor Gail Davies (Nov 2017).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The role of the sponsoring company named information officer (NIO) includes the sharing of animal welfare, best practice and 3Rs related information. The NIO also liaises directly with REDACTED project licence holder network through their own regular meetings, and also raises this type of information and discussion points at the institutional Animal Welfare and Ethical Review Body (AWERB). The licence holder for this work regularly attends both these groups and is also separately aware of the 3R's related work of the 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). Experience to date has shown that 3Rs issues and advances are highlighted, discussed and actions are implemented within REDACTED centrally and effectively via these forums.

Explain the choice of species and the related life stages

Work under this licence will use rats or mice. Animal models cannot yet be replaced in all pre-clinical research because they are required both to answer basic mechanism related questions and to assess the activity of potential new treatments, in complex inter-connected environments which are only found in live animals. Although there are known respiratory and immunology differences between species, the similarities in systems and published knowledge of underlying immunology means that rodents, and mice in particular, are valuable and relevant model systems for the work proposed in this licence.



The animal models used for this work have been chosen to assess mechanisms, and developed to be the least severe and most effective and robust methods of measuring structural, immunological and inflammatory changes. Mice will be suitable for the majority of the immunological work, due to availability of commercial reagents and assays as well as genetically altered animals. Rats will be used where a rat model provides information that translates to human biology more directly than a mouse model. Rats are widely used in safety assessment studies and use of rats will provide a direct comparison with toxicology and drug metabolism data to support a clinical dose calculation.

We will use adult animals. For most of the proposed work we require an established immune system, able to respond to a challenge according to the animal's genetic status. For rodents, this would typically require animals of at least 8 weeks of age. Most animals will not be genetically altered, but we will use genetically altered animals where we wish to investigate the role of genes of interest that might be involved in human disease.





139. Response to novel and emerging human pandemic 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
 - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

No answer provided

Animal types	Life stages
Ferrets	juvenile, adult
Pigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The aim of this project is to support Government and national response to emerging and novel pandemic diseases, human and animal. Enabling them to understand it better and introduce effective control methods.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

REDACTED



What outputs do you think you will see at the end of this project?

This project licence will provide an improved understanding of pandemic infections and their control. The initial objectives is to contribute to the increased understanding of SARS-CoV-2 and mechanisms for its control.

Information arising from the work will be communicated to PHE, the Department of Health and the national Governments. Research findings will be disseminated in the form of papers and presentations.

Positive control sera for use in testing and establishment of assays for research purposes.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The population as whole will be benefit by development of disease-mitigation measures leading to the reduction of the impact of pandemic disease outbreak, as it should reduce both the amount of illness and mortality in the population and economic effect that it has. Benefits for humans and other animals where zoonotic infections are indicated.

How will you maximise the outputs of your work?

Communication to stakeholders, funders, and the general public.

Dissemination to other scientists and collaborators.

Positive control sera will be offered to other organisations undertaking testing and research that require this material

Species and numbers of animals expected to be used

- Ferrets: 96
- Pigs: 20

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Protocol 1 : Vaccination, swabbing, blood sampling and infection with SARS-COV-2

Protocol 2: Injection and blood sampling

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Protocol 1: Animals will experience repeated mild discomfort from sampling or administration of substances, animals may be anaesthetised where the discomfort of the



procedure is deemed to be greater than that of inducing sedation or on safety grounds. In some animals (particularly the virus control groups) clinical disease signs including general signs of illness (inappetence, lethargy, fever) and respiratory signs (cough, sneezing, difficulty breathing) may occur. For an estimated duration of up to 14 days, signs may wax and wane during this period a prodromal or sub-clinical period, variable clinical presentation, resolution periods are anticipated. 'Vaccinated' animals may experience transient pain at the inoculation site during administration and as the local immune response develops.

Protocol 2: Animals will experience mild discomfort from the injections and blood sampling.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Protocol 1: 25% moderate, typically the virus control group 75% mild, typically the intervention groups

Protocol 2: 100% mild

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

A complete biological system is required to study the immunological response to vaccination and challenge, and also for polyclonal antisera raising.

What was your strategy for searching for non-animal alternatives?

None see above

Why were they not suitable?

Cell culture and other techniques can only be used (understanding of virus, development of vaccine) in the build up to the animal experiment to maximise the chances of a successful outcome. Response to vaccination and challenge needs the complete animal's biological system and full immune repertoire.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise



numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Ferret numbers have been estimated based on related research publications, programmes and project licences using ferret vaccination models.

Pig numbers have been based on likely demand for antisera.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The use of a statistically valid minimum number of animals per study will be determined based on expert advice from a professional Biostatistician. Animal studies will be designed in a consistent manner so that inter-study comparisons and data analysis can be performed.

The use of the pig, rather than smaller species such rabbit or ferret means an individual animal will produce more antisera so reducing the numbers required

What other measures apart from good experimental design will you use to minimise numbers?

Animal studies will be designed to maximise collection of biological materials and, where feasible, run in parallel. This will potentially reduce the number of control groups required and therefore increase the data output and research questions that can be addressed. Materials generated during the study will be utilised for in vitro test assays e.g. serum will be harvested to assess immune response and later used as internal controls for other serological assay, limiting the requirement for reagent generation in additional animals. Similarly animal tissue harvested at post mortem.

The number of pigs used for antisera raising will be based on need for the volume of antisera 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

From the SARS-CoV-1 work the ferret is considered the most appropriate model for humans, other than non-human primate.

The pig is used as the protocols are well established for producing relatively large amounts of high titre hyperimmune pig serum against viral antigens



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

They lack the specific biological similarity to human lungs and absence of a complete immune repertoire.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The protocol 1 experiment has its disease-relevant clinical observation criteria and score sheets. No animal will be allowed to progress beyond the described humane end point using a 2-3 times daily monitoring system. On site veterinary teams and animal welfare officers (NVS and NACWO qualified) who participate in each study.

Protocol 2 is for antisera raising, this protocol has already been substantially refined and minimal numbers of injections and blood samples are undertaken and the adjuvant used has minimum adverse effects but still gives a suitable antibody titre.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Best practice information is obtained from NC3Rs, IAT, LASA and the RSPCA. There is frequent knowledge exchange between UK institutions working with ferrets. Publications and articles are also reviewed during the approval process prior to each individual study. Where specialist training is required, inter-institutional exchanges and training visits are organised.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The Establishment is a signatory to the NC3Rs and applies the Culture of Care in animal studies. The Establishment frequently attends or organises external symposia on laboratory animal welfare REDACTED. Staff attending these meetings provide meeting feedback reports locally. In addition, the establishment has a Species Care and 3R Committee where all PILs and animal users are invited to attend. Specialist topics are presented and refinements, such as environmental enrichment, are communicated and opportunities are used for implementation. In addition, specialist knowledge exchange is organised by field and lab exchanges with other organisations, eg PHE and Universities.

The Establishment follows the PREPARE and ARRIVE guidelines

Explain the choice of species and the related life stages

Research into SAR-COV-1 and initial work SARs-COV2 has shown the ferret to be the most biologically relevant hosts apart from non human primates.

For antisera Pigs are the appropriately large size to use minimal numbers of animals for the volume of serum required.

Protocols are well established at the institute for producing high titre hyperimmune pig serum against influenza virus antigens. Similar protocols can be applied for a different antigen, SARS-CoV-2.





140. RoboVOX: studies of soft robotics based devices for the treatment of aero digestive tract disorders

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

No answer provided

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?

We will test prototype devices to replace laryngeal (voice-box) functions of breathing, sound production and swallowing in a large animal model. Experiments will explore safety and potential efficacy for patients with aero-digestive disabilities.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are no good solutions to the problems faced by people with loss of throat functions. Speech, breathing, swallowing are fundamental human activities which require a normal larynx (voice-box). Our vision is to transform the lives of the hundreds of UK patients (1000's EU/US) losing laryngeal function due to cancer (laryngectomees) or other causes. Present routine management of such patients is with a permanent hole in the neck (trachestome) through which to breathe, and a plastic valve between windpipe and gullet through which air can be passed to generate a voice. This voice remains highly limited and is not possible in all patients, plus there are additional quality of life problems around



appearance, psychology, swallowing, taste, smell, coughing, straining, laughing and other basic human activities.

What outputs do you think you will see at the end of this project?

- Knowledge and hypothesis generation relating to mechanisms of integration and host-response to soft robotic airway implants
- Second generation airway implants based on the above for late pre-clinical testing ex vivo and in vivo in a separately funded follow-on study
- Data generation for dossier of clinical trial pre-clinical validation of quality and safety.
- Surgical experience translatable to scaled up animal and clinical work
- Intellectual property generation
- Publications in high impact translational journals as part of TE pre-clinical results in combinations with in vitro basic data.

Who or what will benefit from these outputs, and how?

- Patients, through the delivery of life-changing (and lengthening) technology
- Scientists, through unique knowledge gains in an emergent field and through the development of cross-disciplinary careers for those engaged in this project.
- Society, from a return to society and work of those unable to work due to loss of health and communication.
- IP stakeholders.
- UK plc, through successful commercialisation and the development of a raft of related products for other unmet health needs.

How will you look to maximise the outputs of this work?

- This work will provide data on safety and potential efficacy with respect to a soft robotics Total Laryngeal Replacement. If successful, it will support progression to further, substantive studies of a later prototype.
- Such studies will be informed by our developmental work on surgical and peri-operative processes.
- The present work will generate data for power calculations that will optimise numbers of animals in subsequent studies.
- Observations of device performance in our acute and chronic study will inform the development of a more advanced prototype, including soft robotics, fabrication, signalling and control work.
- The studies will result in knowledge and hypothesis generation relating to mechanisms of integration and host-response to soft robotic airway implants. Such data will inform the development of the broad, emergent field of implantable soft robotics for replacement of lost human function due to genetics, trauma or disease.
- We will continue to explore whether our novel device(s) can form the basis of intellectual property generation and protection. If a device based on this work is eventually commercialised, this would bring revenue to the IP Stakeholders. This in turn would be recycled into future UK-based research gains.
- Where this does not compromise our IP position, we will present and publish our results. We anticipate that large portions of the work we have already performed and will perform will be presented to the scientific community in these ways, regardless of the IP position.
- We have a large multidisciplinary scientific and clinical team. This includes some “rising stars” of laboratory and clinical research. This work forms a further



opportunity for cross-disciplinary learning, career development and the creation of a human resource for the acceleration of UK soft robotics healthcare research for decades to come.

- The Establishment has a cadaver sharing policy; methods of euthanasia include those that may allow unused tissues from this project to supplement other research projects.

Species and numbers of animals expected to be used

- Sheep: 40

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult sheep have been chosen for this project because the anatomy and size of their respiratory tract is comparable to humans, thereby enabling the testing of a human size device.

Typically, what will be done to an animal used in your project?

Protocol 1: Typically animals will be received at least 3 days prior to the procedure, Up to 20 sheep will undergo a non-recovery terminal procedure.

Protocol 2: All animals will be received at least one week prior to surgery to permit acclimatisation and humanisation to prepare animals for the study by training them to allow handling and restraint (eg crate or sling). Long term this will reduce stress during repeated procedures.

Animals will have an operation to implant the total laryngeal replacement device and up to 2 tracheostomies in series with the intact natural larynx. Up to 6 general anaesthetics for the purpose of visualising and testing the laryngeal replacement device or maintenance of the device and tracheostomies / airway will be performed. Up to 30 conscious procedures with local anaesthesia and sedation, if required, will be performed for endoscopy, maintenance of devices and blood sampling. A final assessment will be performed under terminal anaesthesia.

Animal may be maintained for up to 6 months in total.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will experience moderate pain immediately following surgery and will received appropriate analgesia. Pain will be closely monitored using a linear analogue scale appropriate to species.

Uncommon side-effects include wound infections, breakdowns and self-harm at wound sites. Animals will be closely monitored for signs of these and local wound care and antibiotics or repair will be instigated on veterinary advice if required.



It is possible that the tracheostomy will become partially or totally occluded at times, and this would cause increased noise and effort of breathing. This is treated by using suction to remove any obstruction, humidifying the air breathed in by the animals and by removal of a "safety" inner lining tube. Since the laryngeal device above the tracheostomy will be set to the "open" position in between intermittent tests of device functionality, obstructions of the tracheostomy are very unlikely to result in acute, serious obstruction.

We have clearly defined humane end-points, however, should the above measure not relieve 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)?

- Sheep 20 Acute terminal procedures NON-RECOVERY
- Sheep 20 Chronic recovery procedures MODERATE severity

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is ever-increasing scrutiny of the evidence base for safety and potential efficacy of medical devices, fuelled by high profile cases of device failures and increasingly risk-averse university and healthcare systems in the UK. Although we have developed a prototype total laryngeal replacement implant, with proof-of-principle demonstrated on the bench-top, there remain important questions of host response (e.g. local immune response) and quality improvement (e.g. improved biomechanical properties in response to observed challenges in vivo) that cannot be answered other than by animal studies. The present project will generate safety data to increase confidence for further development towards a clinical grade device and inform the design of later iterations of this device and its command and control systems. The knowledge gained herein will impact the whole field of implantable medical robotics, let alone our specific areas of interest, with the potential for disruptive change in the treatment of a wide range of illnesses from incontinence to stroke to facial palsy.

We consider animal use to be unavoidable, therefore, and appropriate in this experimental stage of the project described. This view is supported by preliminary discussions with the UK's regulatory body, MHRA, who have indicated that the present programme of work is a critical step towards possible clinical trials.

Which non-animal alternatives did you consider for use in this project?

We have developed a bench-top rig that replicates air-flow and pressures generated by human lungs and trachea (Figure 5). We are able to tune this to represent changes with



disease and age and have even been able to generate “voice”. This advanced device has significantly reduced the need for animals as many parameters can be tested in the engineering lab instead of in animals, whose patterns of airflow and pressures may vary significantly from those we will face when implanting in humans.

Unfortunately, no appropriate “in silico” computer-modelling of how the device may perform in various human physiological conditions is available to our knowledge. We considered moving directly to human patients undergoing laryngectomy, but the MHRA have indicated caution and the need for the kind of confidence in safety and potential efficacy that only large animal studies may deliver.

Why were they not suitable?

The rig has proved invaluable, but can only replicate biological conditions up to a point, whilst proceeding straight to human is judged premature by our team, our advisors and by the MHRA. No computer model is available.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The present studies are designed to provide data on safety and functionality. As such, there is no formal calculation of numbers required that is possible. 6 animals per study in Protocol 2, is the smallest number that can provide a view on biocompatibility, material safety, and device safety respectively in vivo. This number also permits the use of exploratory statistics for time-series changes in various secondary outcome measures such as blood counts, using each animal as its own control. To account for animal losses through reaching maximum endpoints 1-2 extra animals may be included in the start of the study, this will ensure a suitable amount of contemporaneous data will be collected.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The limited numbers above are appropriate for this preliminary stage in device development and reduce animal use. Samples at different time-points will be assigned identifiers to blind those observing histology, video-endoscopic images and post-mortem analysis. Each animal will be analysed in as many ways as possible without increasing animal suffering potentially to reduce the numbers needed for future experiments (e.g. physiological monitoring, imaging in vivo, analysis of organs) if indicated. We took advice 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?



Cadaver sharing (use of cadaver tissue from other research experiments) will be used to optimise surgery protocols with the aim of reducing the number of subsequent live animal acute studies.

To reduce variation, animals within a study will be ordered in batches from the same supplier and be of similar age / size (likely between 65 and 85 kg at implantation)

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pigs and sheep tolerate large airway surgery extremely well, with minimal morbidity, and are regularly employed in physiological studies and preclinical testing of airway therapeutics and devices. This has been demonstrated by extensive published work on laryngeal transplantation and by our preliminary cadaveric animal studies.

Smaller animals would not tolerate testing of our device sleeve due to the necessary wall thickness of the material used. It is not possible to miniaturise the soft robotics device to a size applicable to smaller animals (with present technological state-of-the-art), and their airway dynamics are orders of magnitude different from those of humans.

Based on cadaveric studies and suitability of the animal temperament to allow conscious endoscopy procedures, sheep have been selected as the appropriate model for the recovery studies.

Recovery studies will be conducted with the native larynx intact, this will allow biocompatibility testing and functionality testing of the device whilst maintaining normal eating and swallowing, thus allowing the complications associated with gastrostomy to be avoided.

Why can't you use animals that are less sentient?

Smaller animals would not tolerate testing of our device sleeve due to the necessary wall thickness of the material used. It is not possible to miniaturise the soft robotics device to a size applicable to smaller animals (with present technological state-of-the-art), and their airway dynamics are orders of magnitude different from those of humans.

Acute terminal models will not allow long term questions of host response (e.g. local immune response and impact of biofilm), quality improvement (e.g. improved biomechanical properties) and biocompatibility and safety to be tested.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Published, research into physiological and immunological responses to laryngeal transplantation and laryngeal nerve grafting in pigs has permitted the development of



optimised pre-operative (e.g. acclimatisation and humanisation), anaesthetic and post-operative (e.g. humidification and tracheostomy management) protocols to optimise animal welfare and minimise suffering. This experience will be applied to the sheep model.

Animals will be trained prior to surgery, to co-operate and tolerate procedures, thus minimising stress during handling for observation / conscious procedures. Training may include clicker training and/or food rewards.

Insertion of cannula / vascular access ports will allow repeat blood sampling and anaesthesia induction using minimal restraint and stress techniques.

Insertion of up to 2 tracheostomy tubes alongside the device will allow repeat anaesthesia to be conducted with minimally invasive procedures via one tube. The second tube will allow endoscopy / cleaning of the device.

Post-operative care will include regular monitoring, including overnight care where necessary to ensure animal recovery is optimal and welfare of the animal is maintained.

Surgical expertise at the Designated Site further enhances animal welfare, by providing close collaboration with dedicated large animal surgeons and veterinary anaesthetists, and ready access to advice in the case of unforeseen complications or intercurrent illness. For clarity on care in such circumstances, we will refer as appropriate to the NVS, in the first instance.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We refer to our previous publications where we describe anaesthetic and care protocols for animals undergoing large airway surgery, including tracheostomy and gastrostomy care. These were based on a decade of experience. We are not aware of other published guidance in this area of experimentation.

Veterinary anaesthetists work to best practice guidelines for large animal anaesthesia and maintain CPD to keep up to date with new practices.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

NC3R website will be used as a source of information of advances in 3Rs, as well as review of the regular updates received from the designated establishment. Any advance considered appropriate in this PPL will be incorporated into the in-vivo experiments where possible.



141. RUNX, MOZ and associated gene functions in normal tissues and as therapeutic targets in cancers

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Mice	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 is the aim of this project?

The genes encoding the AML1/RUNX1 transcription factor and the transcriptional co-activator MOZ are critical for haematopoietic development and are frequently rearranged or mutated in human leukaemia. The over-reaching aim of this project is to investigate the functions of RUNX1, MOZ and their associated genes, in haematopoietic development, maintenance and malignancies. The project aims to understand how alterations in these genes and their functions lead to malignancies and in doing so potentially identify new therapeutic targets.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is estimated that one out of 2 persons born after 1960 will be diagnosed with some form of cancer during their lifetime and although treatments have dramatically improved, still half of the patients do not survive their disease for more than 10 years (source: CRUK



website). Leukaemia is the 13th most common cancer in the UK, with around 10,000 new cases diagnosed and around 4,600 deaths (representing 3 % of all cancer deaths) in 2016. Treatments of Acute Myeloid Leukaemia (AML) have not dramatically changed, or improved, for the last 30 years. The current treatments are very debilitating, associated with high morbidity and inappropriate for a large fraction of patients. Therefore, there is a pressing need for the development of more specific and efficient therapies.

What outputs do you think you will see at the end of this project?

Understanding how genes control the generation and maintenance of blood cells. New Insights could be translated in in vivo generation of blood cells required to treat cancer patients by transplantations or with immunotherapies.

Elucidating function of these genes studied in development of disease, specifically haematological malignancies (leukaemia, lymphoma).

Identification of novel therapeutic targets for treatment of blood disorders and cancers.

Rationale for generation of new drugs by drug discovery teams and pharmaceutical companies.

The research findings will be published in high impact peer reviewed journals and will be of interest to clinical and academic scientists. In addition, our work programme has clear implications for the treatment of patients with genetic blood diseases or patients suffering from leukaemia. The new findings generated could lead to defining new strategies to generate human blood generating cells (haematopoietic stem cells, HSCs), critically needed for bone marrow transplantations for complete cure of leukaemia.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The main beneficiaries will be other scientists, health professionals and pharmaceutical companies as well as patients with leukaemia. We anticipate that patients won't be able to benefit from this work within the time frame of this project licence.

How will you maximise the outputs of your work?

On a weekly basis the REDACTED.. In addition, laboratory members will attend national and international scientific conferences which are both discipline-specific as well as cross-disciplinary and are attended by the full range of academic, clinical and commercial scientists. We will also strive to ensure that the research performed will be published in high quality, peer reviewed journals of international standing. The laboratory has an excellent track record for this REDACTED. Data will be published in international open access scientific journals, made available online through REDACTED, as soon as possible. Publications in high quality journals will improve the laboratory's capacity to recruit and train future scientists.

Press releases relating to findings arising from the proposed research for local and national mass media will be managed by dedicated press agents within The REDACTED. We also regularly organise open days, school days and laboratory tours where fundraisers, patients and their families and school children can come into the laboratory and at these events, we explain our research to them.



One significant potential outcome of our work is the identification and validation of new therapeutic targets for leukaemia. In order to explore and enhance any potential therapeutic benefits of our research, we will meet twice a year with the onsite Business Development Manager responsible for the management of Intellectual Property (IP). Concrete plans to develop industrial collaboration, involvement with Research Technology development laboratories or engage third parties will be devised as findings emerge.

Species and numbers of animals expected to be used

- Mice: 4,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

A typical investigation of the role of a gene in blood cell development and/or maintenance would involve the use of genetically altered mice and their wild type counterparts as controls. In some instances, there may be evidence of the gene being essential for development and therefore, embryonically lethal. In such situations, no lasting distress or suffering to the animals is anticipated. However, in order to overcome these lethality outcomes, there may be a requirement to introduce compounds in mice in order to induce these genetic alterations conditionally in specific tissues/cell types. Additionally, agents may be administered to evaluate the functional consequences of these genetic alterations, such as the ability of the stem cells to divide and the evaluation of genetic /signalling pathways critical to generate blood cells. In some instances, cells will need to be transplanted to evaluate their functional capacity with or without these genetic alterations. To this end, mice may be conditioned prior to transplantation either by administration of immunosuppressive drugs or by exposure to irradiation. For studies involving mice with reporter genes associated with these alterations, mice may be imaged under recovery anaesthesia throughout the study. In order to obtain experimental readouts animals, will have blood micro-samples taken from superficial veins.

Leukaemia is not associated with pain during the period in which we conduct our observations. Disease will be monitored regularly by either using blood sampling from peripheral vessels or by imaging methods such as fluorescence under brief general anaesthesia. For some procedures that involve surgery under general anaesthesia, we will administer pain killers and monitor the mice closely during recovery. At the end of an experiment or when an experimental goal has been achieved earlier than anticipated, the mice will be humanely culled, either with or without preceding terminal bleeds under non-recovery anaesthesia.

Mice will be group housed in ventilated cages which have their environment enhanced with items such as tunnels, houses, nesting material and gnawing blocks.



Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Most procedures in the protocol, such as irradiation of animals, blood sampling or injection of cells and compounds are not associated with significant side effects. Mice developing leukaemia or solid tumours exhibit signs of disease, such as hunched posture, “goose-bumps”, piloerection and poor levels of socialising and interaction. In more progressed models of the disease animals may display signs of anaemia and hind limb paralysis. Under these circumstances, and whenever else an animal displays features of ill health, or at the end of each experiment, mice will be humanely euthanized using a Home Office sanctioned 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 species)?

The vast majority of mice are only expected to experience the mildest clinical symptoms due to leukaemia before being humanely killed. Additionally, some mice will experience the discomfort of repeated administration of therapeutic agents. We will aim to utilise the least stressful route of administration and the smallest of volumes wherever possible.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 genes associated with normal development that are also dysregulated in cancers will be researched as far as possible using non-animal methods. However, the simplicity of the laboratory- based systems relative to the more complex animal model wherein the interactions of different cell types within tissues is intact and similar to the human body, renders it unsuitable to draw concrete scientific conclusions.

What was your strategy for searching for non-animal alternatives?

We perform pilot experiments with laboratory based generated cell lines, established cancer cell lines, or primary samples, to evaluate the potential roles of the candidate genes of interest. Once we have obtained results that support and inform us on the potential role of these genes, we will be able to design appropriate animal experiments.

Why were they not suitable?

Currently, there is a lack of a non-animal system that can accurately model the homeostasis of complex haematological organs and epithelial tissues or the development of cancers in these tissues. This is because these events take place in very complex



cellular architecture, involving interactions between many different cell types which cannot be reproduced in laboratory-based experiments. Therefore, without the use of a live, whole animal experimental system, the biology of haematological and epithelial homeostasis and malignancies cannot be meaningfully studied.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Data available from the literature or from pilot studies conducted in our laboratory are used to perform power analysis to determine an appropriate sample size for the definitive experiment. In general, we will use a sample size capable of detecting a 40% practical difference with 80% power and 95% confidence.

Based on past experience, group sizes of between 3 and 12 animals (dependent on the readout, fewer for transplanted leukaemia compared to spontaneous leukaemia in Genetically Modified (GM) mice) per experimental group suffice.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

In previous studies, power analysis was utilised to determine the minimum number of animals required per experimental group. This helped reduce utilisation of unnecessarily large groups of animals other than those needed to achieve the experimental goal.

Wherever possible, alternatives to culling at defined time points such as use of live bioimaging or repeated blood sampling to follow disease development and response in real time were used.

In addition to the use of laboratory-based systems, wherever possible, pilot experiments were carried out to test for the extent of an expected phenotype prior to a full scale confirmatory experiment. For disease models, the cryopreservation in multiple aliquots (collection and preserving of samples) of normal and humoural cell populations and samples, eliminated the requirement for continuous production of cohorts of mice.

What other measures apart from good experimental design will you use to minimise numbers?

Pilot studies will be performed if applicable and, after analysis of the results, group sizes for subsequent experiments will be determined based upon these data. As far as possible, multiple parameters will be evaluated in a single mouse. Live imaging of the same animal at multiple time points also greatly reduces the numbers required. Whenever possible, tissues and samples from related studies may be acquired from other groups for analysis before carrying out large scale studies of our own.

Refinement



Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice have been chosen for the study because they represent the least sentient species from which meaningful experimental data can be generated, while exhibiting considerable genetic and biological similarities to humans with regard to their blood forming system. Only a mammalian model system has the potential to accurately mimic both the anatomy and complex cell biology, including normal and malignant tissues and the associated microenvironmental interactions, of the human counterparts. Furthermore, there is considerable experience in the wider scientific community regarding the use of mice as a model system for human malignancies and many reagents exist for the phenotypic characterisation of mouse cells.

General welfare and humane endpoints will be defined and adjusted according to the guidelines published by Workman and colleagues (BJC 2010, 102, 1555-1577). The techniques used have been carefully evaluated to minimise distress to the animals. Mice used in surgical procedures will receive anaesthesia, analgesia and post-operative rehydration by subcutaneous injection, followed by careful observation. In other areas, irradiation doses will be administered at a level sufficient to induce bone marrow suppression but no other long term consequences; intra-femoral injections will be not be performed routinely, only where the scientific justification is high; and in studies that result in the initiation of malignancy, animals will be closely monitored for health status and killed by a Home Office approved method when signs of ill health are displayed. In all Protocols, best practice will be followed in the administration of substances and the removal of body fluids (Morton et al. Laboratory Animals (2001) 35, 1-41).

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Less sentient animals do not have a haematopoietic system. Mice are far more similar to humans than other animals possessing lungs e.g. birds or reptiles and this is critical both for using reagents like drugs developed for human targets and for translating findings to the clinic. Cancers develop over many weeks to months, so use of terminally anaesthetised animals or immature animals is not practicable. Also, immature mice lack a functional immune system which is desirable in cancer research.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The techniques used have been carefully evaluated to minimise distress to the animals. Mice used in surgical procedures will receive anaesthesia, analgesia and post-operative rehydration, followed by careful observation. Animals will be monitored closely and carefully especially for pilot studies involving the administration of novel substances. In other areas, irradiation doses will be administered at a level sufficient to induce bone



marrow suppression but no other long term sequelae. Intra-femoral injections will be not be performed routinely, only where the scientific justification is high. In studies that result in the initiation of malignancy, animals will be closely monitored for health status and killed by a Home Office approved method when signs of ill health are displayed.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Relevant published literature will be used as template for experimental design and decision making (Workman et al., 2010. Guidelines for the welfare and use of animals in cancer research. BJC, 102, 1555-1577).

We will follow guidelines of good practice [Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, et al. British Journal of Cancer, 102:1555-77 (2010)]. 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.

Guidelines for Body condition score. [Ullman-Cullere, Lab Anim Sci. 1999 Jun;49(3):319-23]

How will you ensure you continue to use the most refined methods during the lifetime of this project?

By reading 3Rs literature and participating in 3Rs workshops locally and nationally. Through discussing refinements with our NACWO, NVS and HO inspector.

Explain the choice of species and the related life stages

Mice have been chosen for the study because they represent the least sentient species from which meaningful experimental data can be generated, while exhibiting considerable genetic and biological similarities to humans with regard to their blood forming system. Only a mammalian model system has the potential to accurately mimic both the anatomy and complex cell biology, including microenvironmental interactions, of normal and malignant human haematological organs and epithelial tissues. Furthermore, there is considerable experience in the wider scientific community regarding the use of mice as a model system for human malignancies and many reagents exist for the phenotypic characterisation of mouse cells.

One of the over-arching aims of the project is understanding the contribution of master regulators and their associated genes in blood cell development and in tissue homeostasis (stability). A second goal of the project is the long term applicability of these findings in delineating mechanisms of disease progression. To address this, all life stages need to be utilised. Specifically, embryonic and neonatal stages will need to be employed for studies focusing on the development of blood generating cells. For studies aimed at understanding disease progression, mostly juvenile and adult forms will be utilised. In some studies, a combination of various stages may also be necessary, for example, cells from foetal stages may need to be harvested for transplantation in adult mice.





142. Redacted

Project duration

5 years 0 months

Project purpose

Basic research

Key words

No answer provided

Animal types	Life stages
REDACTED	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 is the aim of this project?

We aim to determine whether angiogenic mechanisms within the REDACTED pituitary mediate melatonin regulation of seasonal REDACTED.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We will investigate the processes underlying the adaptation of animals to a changing environment. The project is based on our recent discovery that angiogenic mechanisms within the pituitary gland respond to seasonal changes in day length, i.e. photoperiod, generating pro- or anti-angiogenic isoforms of VEGF-A at different times of year. The differential expression of VEGF-A isoforms is regulated by the duration of melatonin exposure at night, which is the translator of photoperiodic information, and is predicted to result from alternative splicing of the VEGF-A gene, leading to seasonal remodelling of the vascular connections within the pituitary and paracrine regulation of pituitary endocrine networks that REDACTED annual cycles in reproduction, hair growth and other functions. Therefore, these studies are applicable to all animal species that have developed annual physiological cycles as a strategic means to ensure survival in a changing environment. In particular, the project is directed towards REDACTED that display robust seasonal cycles



in their physiology but findings could also be extended to other animals such as horses. A better understanding of their processes of adaptation will ensure improved efficiency in the farming and, potentially, equestrian industries. The impact of these studies extends to human health, in particular to the understanding, prevention and treatment of illnesses derived from day/night shifts in work schedules and trans-equatorial flying. Moreover, the impact can extend to the treatment of other diseases that show seasonal incidence such as certain types of depression and cancer. A key objective of this project is to unravel the way in which the photoperiodic decoder, i.e. the duration of nocturnal melatonin output throughout the year, can induce alternative splicing of the VEGF-A gene to generate the desired adaptive biological response (induction or repression of oestrus).

We will tackle this vital question using animal models that exhibit annual physiological cycles at a given season, using *in vitro*, *ex vivo* and *in vivo* strategies. If the molecular and cellular signalling mechanisms underlying the adaptation to a changing environment can be more clearly understood, then both artificial and natural regulators of seasonal physiological cycles may be developed and REDACTED, and pathologies with seasonal incidence may be more effectively overcome. For instance, foodstuffs that regulate alternative splicing have been identified (e.g. broccoli, carrots, olive oil), and although much of the current work has focused on the effects of high fat diets, understanding the regulation of micro-vascular remodelling and intra-pituitary paracrine REDACTED by alternative splicing of VEGF may help us to recognise how, for example, different farming and breeding regimens could be implemented to improve the REDACTED of REDACTED in REDACTED (and potentially, horses, for instance how novel photoperiodic strategies could be used to enhance the welfare of horses subjected to trans-equatorial travel for breeding or competition purposes), and how the occurrence or recurrence of clinical conditions in humans (e.g. prostate, breast and ovarian cancers, which are linked to melatonin production and VEGF production) could be better prevented and/or treated more effectively.

What outputs do you think you will see at the end of this project?

We would hope to determine the mechanisms of photoperiodic signal transduction involved in the adaptation to seasonal changes in the environment. It is well established that in photoperiodic species the duration of melatonin secretion from the pineal gland during the night translates the effects of day length on seasonal physiology, and that the pars tuberalis of the pituitary gland is the primary site of action of melatonin. Recent work *in vitro* has shown that the duration of melatonin exposure within the pituitary can induce alternative splicing of the VEGF gene, resulting in different outputs of VEGF isoforms, and that these isoforms can alter the production of prolactin and gonadotrophins. Similarly, the hypothalamic-pituitary portal system undergoes annual remodelling associated with the seasonally regulated differential expression of VEGF isoform output. Therefore, this appears to be a mechanism for seasonal adaptation to changes in the environment whereby the transducer (i.e. the melatonin signal) of an environmental independent cue (i.e. day length) is decoded by an angiogenesis regulatory system that will affect not only the vascular connection between the hypothalamus and the pituitary gland, but also the function of at least two pituitary endocrine systems that regulate seasonal physiology. Moreover, preliminary work has shown that a similar mechanism operates in the horse, providing evidence that this could be a conserved mechanism for physiological adaptation to a changing environment. However, all work so far has been conducted either *in vitro* or *ex vivo* (using specimens collected from a commercial abattoir) and, therefore, an *in vivo* study is needed to demonstrate that indeed this system of adaptation operates in living animals.



We anticipate outputs that will include new publications on REDACTED of pituitary vasculature, presentations at endocrinology meetings, such as the annual meeting of the Endocrine Society in the US, engagement with the public on its physiological significance (previous work was reported in the national press), and potential for additional funding for interventions that could modify angiogenesis in the pituitary to modify seasonal physiology.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The outputs of this research will benefit the scientific community in the first place and ultimately the general public. Benefits to the scientific community will be derived from the clear understanding of how annual time is measured by animals that have adapted their seasonal physiology to the temporal changes in the environment. Although it has been well established that the pattern of melatonin secretion from the pineal gland translates the effects of day length (photoperiod) on the annual reproductive cycle, the mechanism for melatonin signal decoding has just begun to be elucidated.

An 'in vivo' demonstration of the proposed decoding mechanism will provide convincing evidence to the scientific community that what has been shown 'in vitro' and 'ex vivo' occurs in living animals to enable them to adapt their physiology to the predictable annual photoperiodic cycle. The first output is expected for the spring of next year, when we anticipate communicating the preliminary results of this study at the annual meeting of the Endocrine Society (USA). Full publications reporting the results of this work are not expected before the end of the project in March 2022. The first publication of the 'in vitro' work related to this topic received extensive coverage by the national and international press, so it is possible that the results of the proposed 'in vivo' experiments will continue to be of interest to the media and reach a wider audience. Because it has been demonstrated that the melatonin duration-induced differential expression of VEGF isoforms within the pituitary gland affects not only the gonadotrophic axis but also the lactotrophic axis, and that this appears to be a conserved mechanism of adaptation across species, we anticipate that other pituitary endocrine systems, such as the ACTH axis to regulate the response to stress, and TSH axis to regulate metabolism, are likely to be under the same mechanism of REDACTED. Therefore, in the long term, the outputs of this study are likely to benefit the general public, as the information gained may be applicable to the treatment of ailments derived from insufficient light exposure, constant air travel, shift work, etc. That being the case, we also anticipate collaboration with industry for the production of drugs designed to alleviate those ailments.

How will you maximise the outputs of your work?

We will communicate through conferences, publications, press releases, interviews, and articles in the farming press.

Species and numbers of animals expected to be used

- REDACTED: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals receive either a subcutaneous implant and once daily injections, or twice daily injections for a maximum of 21 days. Blood may be sampled up twice weekly to determine hormone status, after which we will insert an indwelling cannula into the jugular vein (under local anaesthetic using aseptic technique) for one day. Small amounts of blood are sampled from this cannula up to a maximum of 60 times.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

There are no expected adverse effects apart from a small needle prick when the injection goes in and the cannula is inserted.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Mild for all animals

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

REDACTED of REDACTED in REDACTED is down to a complex interplay between the brain decoding light signals to tell the animal what season it is, the pituitary gland interpreting the signal, the blood vessels that carry the hormones around the body and the interaction of the hormones on the reproductive tissues. To understand how this is REDACTEDled requires studying this interplay in live animals.

What was your strategy for searching for non-animal alternatives?

We have undertaken extensive experiments on cells grown in culture to find out the proposed mechanisms and these experiments are designed to find out if the previous experiments in a dish are representative of real life in an intact animal.

Why were they not suitable?



They provide information about the potential molecular mechanisms, but we cannot find out if they a) happen in intact animals and b) are sufficient to change the physiology of the whole animal. To do this requires experiments in intact animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We are measuring hormone concentrations in REDACTED, and there is inevitably some variation between groups. However, we have modelled the numbers that we need in order to discover whether the changes we see will be sufficiently robust to be able to draw conclusions using standard statistical modelling programs. This helps us have confidence that the numbers we use are the minimum needed to obtain a reliable result, so that not to have to undertake additional experiments on animals.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We used a qualified statistical consultant, as an advisor to the project.

What other measures apart from good experimental design will you use to minimise numbers?

This is a very simple experimental design, but all tissues will be banked and will be used before further experiments take place, wherever possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The experiment is designed to be minimally invasive (subcutaneous implant then daily intra-muscular injections, blood collection from a vein up to twice a week, followed by a cannula on the final day).

Local anaesthesia will be used for cannulae implantation and securing to the neck with a suture

Animals will be in sight and sound of other animals during the procedures and trained with positive re- reinforcement (small quantities of food) during protocols.



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

REDACTED are farmed seasonal breeders and the species that we are trying to identify the changes in. Mice and rats, and non-mammalian species are not valid animal models for the purpose of the study.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The procedures are as minimally invasive as possible. We will use local anaesthesia to implant catheters. Aseptic implantation of catheters with clean technique for blood sampling by trained and competent licence holders and technical staff and single use needles all minimise harms. Total blood samples obtained will be according to published guidance

<https://www.nc3rs.org.uk/blood-sample-volumes>

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Guidelines for the Housing of REDACTED in Scientific Institutions (PDF). NC3Rs guidelines for experimental design. LASA guidelines for blood sampling.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We work with the REDACTED, the NC3Rs and experts in the field to ensure that further refinements can be made wherever possible.

Explain the choice of species and the related life stages

The purpose of the project is to understand how seasonal REDACTED is REDACTEDled in ewes, therefore we need to use adult ewes that are fertile.





143. Sensory and spatial processing in the mouse cerebral cortex

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Mice	juvenile, adult, 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 is the aim of this project?

This project investigates how information from different sensory modalities and spatial information are integrated in the mouse cerebral cortex in order to build representations of the outside world and to form memories. It also addresses how these processes are altered in certain neurodevelopmental disorders and in neurodegeneration.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Of necessity most of neuroscience research in the past has focused on 'lower' cortical areas and relatively simple stimulus processing in anaesthetised animals. In order to really find out how the brain works one needs to study how different sensory inputs are integrated with internal representations of the world, and how the outcome relates to a meaningful behavioural response. The as yet understudied retrosplenial cortex is ideally placed to study these issues, but it is also one of the first brain areas to show deficits in Alzheimer's disease. Understanding the normal function of this area will therefore be an



important foundation for testing potential treatments in mouse models of Alzheimer's disease.

What outputs do you think you will see at the end of this project?

The proposed research will add to the growing body of knowledge about how interconnected brain regions interact to encode and store (visuo-spatial) memories and how this varies according to the types of cue that are being used. Furthermore, we hope to establish how a specific cellular pathway known to be involved in the regulation of blood pressure is associated with cognitive decline in Alzheimer's disease and how drugs targeting this pathway can reverse memory loss. Finally, we hope to find out how different visual areas of the (mouse) brain work together in order to encode different aspects of the visual world, and how this process is disrupted in mouse models of the developmental disorder REDACTED. Primary outputs for all results of our research will be publications in international, peer-reviewed journals.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

While the most immediate beneficiaries will be other researchers with an interest in brain circuits supporting memory as well as in experience-dependent plasticity of sensory processing there are a number of other likely beneficiaries in the longer term. The work on the rRAS pathway in mouse models of AD is ultimately aimed at delivering novel therapeutic options to patients with Alzheimer's disease for whom there are currently very few treatments available, and none that address the root causes. The work on mouse models of REDACTED will ultimately benefit patients suffering from this condition as it will lead to greater understanding of the neural deficits which will in turn be a prerequisite for developing treatment strategies. Results of the research will be shared at the annual REDACTED Day organised by the REDACTED.

How will you maximise the outputs of your work?

Outputs of this work will be maximised by extensive collaboration. Work addressing objectives 1, 2 and 3 (visuo-spatial memory engrams and integration of sensory and motor cues in the RSC) will be done in collaboration with colleagues in the psychology department. Work addressing objective 4 (modulation of rRAS pathway in mouse models of AD) will involve collaboration with researchers in two other universities. Objectives 5 and 6 will be addressed in collaboration with colleagues working in neuroscience and in mental health at my REDACTED.

Knowledge will be disseminated by publication in leading journals in the field as well as through talks and posters at national and international conferences (e.g. British Neuroscience Association, Forum of European Neuroscience, European Visual Cortex Meeting), including both positive and negative results.

Species and numbers of animals expected to be used

- Mice: 2210

Predicted harms



Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mice will be raised either in a standard lab environment or their sensory experience will be manipulated in some way (such as raising in darkness or monocular lid suture). They will be trained on sensory discrimination or spatial memory tasks. They will have fluorescent substances injected into the areas of the brain under study, and will have a cranial window implanted under general anaesthesia. Their brain activity will then be imaged while they will be carrying out the tasks that they have previously been learning. Imaging will be repeated over the course of several weeks. In the end the animals will be humanely killed.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Animals are expected to experience short-lived (up to 48 h) post-surgical pain which will be controlled by analgesics. Animals are expected to exhibit some weight loss during the training on visual discrimination and spatial memory tasks which is likely to last for 2-3 weeks and will be carefully monitored.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

The expected severity for all animals subjected to experimental procedures which include surgery under general anaesthesia is expected to be moderate. The expected severity experienced by animals bred for the maintenance of genetically altered lines is expected to be sub-threshold or mild in virtually all cases, very rarely moderate.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 processing of sensory and non-sensory information by the brain about the outside world, the formation of memories, and the influence of experience-dependent plasticity as well as genetic factors can at present only be studied in intact animals which can integrate sensory experiences over time and produce behavioural responses in return.

What was your strategy for searching for non-animal alternatives?



I have considered both in vitro and computer modelling approaches.

Why were they not suitable?

It is impossible to maintain a whole brain and sense organs alive in isolation in order to test true circuit function, and even if this were possible, we would be lacking a behavioural readout.

Neurocomputational approaches have helped us in the past to interpret results obtained in vivo. However, real data collected in vivo are needed to feed into any models, and for the questions addressed here, such data are not available.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Statistical analyses will be based on comparisons between cohorts of experimental animals (e.g. animals which have been raised in a specific way) and control animals (e.g. normally reared animals).

Calculated experimental group sizes in order to achieve a desired statistical power depend on effect size. This can only be estimated here; we used Cohen's d as a standardised effect size (representing the difference between treatment and control means, calibrated against variability, see EDA). For a medium effect size and a power of 0.8 the G*Power software gives group sizes of 24 for comparison between two independent groups (means). Based on past experience we need to take into account a rejection rate of 20% (for insufficient quality of data etc.) and therefore estimate 30 animals per group.

On this basis we have calculated the following numbers of experimental animals.

Objective 1

(To elucidate what the retrosplenial spatial memory "engram" represents):

3 groups = 90 animals in Protocol 2

Objective 2

(To determine the contributions of visual and non-visual cues to engram formation)

9 groups = 270 animals in Protocol 2

Objective 3

(To elucidate how visual and locomotion signals are integrated in RSC during spatial memory acquisition)

3 groups = 90 animals in Protocol 2



Objective 4

(To elucidate the role of rRAS activation on spatial memory processing in the RSC and cerebrovascular function in mouse models of AD)

3 groups = 90 animals in Protocol 1

groups = 90 animals in Protocol 2

Objective 5

(To determine which visual functions are supported by which of the visual cortical areas in the mouse, and to which extent they are subject to experience-dependent plasticity)

groups = 120 animals in Protocol 1

4 groups = 120 animals in Protocol 2

Objective 6

(To elucidate how mutations of the CACNA1C gene affect experience-dependent plasticity and visually driven behaviour)

4 groups = 120 animals in Protocol 1

4 groups = 120 animals in Protocol 2

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Chronic (longitudinal) imaging will be used throughout this project, an approach that reduces the total number of animals both by obtaining more data from each animal and by the increased statistical power of paired over non-paired comparisons. For example, with only two time points at least twice as many animals would be needed with acute assessments, three times as many for three time points. Consequently, many more animals would have to be killed to achieve the same statistical power. Experimental design has been optimised based on many years of experience in my own lab and by drawing on the experience of others working in my or similar fields, as well as by participating in the REDACTED. I have also drawn on the NC3Rs EDA (Experimental Design Assistant).

What other measures apart from good experimental design will you use to minimise numbers?

Apart from experimental design, the second-most important factor in the reduction of the number of animals used will be efficient breeding. This includes optimising the breeding of genetically altered REDACTED in terms of crossing the right animals to achieve the maximum number of offspring of the desired genotype, as well as ensuring that the number of animals bred and the timing of their births allows the maximum to be used for 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.



Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

For this project we will use both juvenile and older mice, and the principle method of assessment will be in vivo imaging of brain activity. The assessment itself is pain-free, and if properly habituated, the animal should not experience any distress or suffering. Pain will potentially be experienced following surgery to implant the cranial window through which imaging is performed. However, this surgical intervention is carried out under general anaesthesia, and the risk of pain will be minimised by the use of analgesics.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Only mammals have a cerebral cortex and therefore the neural circuitry whose function and plasticity will be studied in this project. Mice are the least sentient species in which visuospatial memory and visual experience dependent learning similar to that in humans has been observed. The work can not be carried out in terminally anaesthetised animals since these would not be able to engage in behavioural tasks, and higher cortical areas would be silenced by anaesthesia.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals will be monitored daily post-operatively (for at least 5 days) using an approved score sheet. Animals will be given analgesics at the beginning of any surgery and post-operatively. Animals trained on specific tasks will be habituated gradually to the experimental setup and the tasks. If trained animals show signs of distress or otherwise stop engaging in a task the session will be terminated and the animal returned to its cage.

We will endeavour to reduce the use of water control, for example by increasing the hedonic value of the reward (by adding saccharine to the water) where this is possible without compromising the engagement of the animal with the task. We will follow national guidelines drawn up by the NC3Rs working group on refining behavioural training of rodents for high-yield sensory discrimination tasks.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

REDACTED In addition, we follow guidance (SOPs) issued by the NVS. REDACTED, the technique central to this project. The working group will issue best practice guidance in due course, which will then be followed for this research.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

At REDACTED level, we are regularly updated on advances in the 3Rs by the HOLC/NTCO as well as the NACWOs and the NVS. These will be implemented during the project as soon as possible. In addition, we will attend an annual 3Rs symposium at which individuals from the participating universities showcase their 3Rs initiatives. I will



implement any guidance issued by the NC3Rs working group on refining behavioural training of rodents for high-yield sensory discrimination tasks as soon as possible.

Explain the choice of species and the related life stages

Mice are mammals, like humans, and their brains are organised and function in a similar, albeit simpler way. Of all mammalian species they are the easiest to genetically alter, which allows us to study neurodevelopmental conditions and neurodegeneration as well as to label specific populations of neurons. We will use both young, adult and ageing mice in order to study perceptual and learning processes throughout the lifespan and to analyse the effects neurodevelopmental conditions and neurodegeneration.





144. REDACTED-mediated control of vascular function

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants.

Key words

No answer provided

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

Blood vessels contain an inner layer known as the vascular endothelium. This consists of a single layer of endothelial cells which maintain healthy blood flow and vessel function. The endothelium acts in part as a barrier to regulate transport of substances into the surrounding tissue. Excessive inflammation of blood vessels at specific locations can lead to mechanical damage that eventually causes heart attack or stroke. Therefore, identifying the mechanisms by which endothelial cells maintain healthy blood vessel function is important to devise new medicines to prevent disease.

This project aims to examine how REDACTED, an intracellular protein, regulates two important properties of endothelial cells that are required for healthy blood vessel function; mechanoprotection (=the cell's ability to protect itself from mechanical stress due to increased blood flow) and angiogenesis (=formation of new blood vessels). We believe that REDACTED's effects are mediated in part via interaction with another protein called "REDACTED". REDACTED is essential for maintenance of "caveolae" (=bulb-shaped invagination in the cell membrane) at the endothelial cell surface, and which flatten to protect endothelial cells from damage following mechanical stress. Caveolae also control angiogenesis as they contain numerous proteins involved in blood vessel formation. Therefore, we predict that the loss of REDACTED will cause a reduction in cell surface caveolae and, as a result, reduce the ability of the endothelium to protect itself following mechanical stress and control angiogenesis.



This project will test this idea using genetically modified mice in which key proteins of interest (REDACTED, caveolin-1[=the main component of caveolae]) have been deleted, and characterise the resulting effects in whole animals and in isolated tissues using cutting-edge imaging techniques.

Mechanical stress will be induced by injection of anaesthetised mice with an optimised dose of dobutamine, a drug which rapidly increases cardiac output, to induce mechanical stress in small blood vessels supplying the heart. 1-minute post-dobutamine treatment, mice will be humanely killed for removal of the heart tissue which will be used to measure blood vessel integrity and levels of key proteins. Some mice will also be transferred to a secondary establishment, where they will undergo the same procedure as described above but with an additional step in which sedated mice will be injected 1 hour prior to dobutamine treatment with substances which enable visual measurement of small blood vessel integrity. This will be achieved using specialised imaging equipment only available at the secondary establishment.

To assess angiogenesis, anaesthetised mice will be injected with ice-cold Matrigel. This solution contains factors that promote angiogenesis and allows us to measure formation of new blood vessels in living mice. After 7-10 days, the mice will be humanely killed via a Schedule 1 method prior to removal of the Matrigel plug for analysis of angiogenesis by microscopic visualisation of new blood vessels.

We anticipate publishing the results of this research programme in high impact open access journals. The animal work carried out as part of this licence will be reported in accordance with the ARRIVE guidelines in order to allow transparency and ensure that our findings can be reproduced, thereby minimising unnecessary follow-on animal experimentation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

Data from our experiments will lead to a better understanding of how REDACTED, a protein which suppresses blood vessel inflammation, can protect the endothelium from mechanical damage and control the formation of new blood vessels through controlling expression of caveolae. This knowledge will be invaluable for researchers studying fundamental processes important in endothelial cell biology, cardiovascular disease and vascular injury. Another potential benefit would be validation of the REDACTED/REDACTED interaction as a viable target for developing new drugs for many cardiovascular diseases.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Mice are well established as the model system of choice for identifying the function of specific genes in controlling the functioning of the vascular endothelium in mammals. This is achieved by deleting the relevant target gene, and observing the consequent effects both on the mice and cells or tissues derived from them. We anticipate using



approximately 900 mice over a five year period. This represents the minimum necessary and will be achieved using methods of analysis that rely on small numbers of cells, for example microscopy-based measurements of the number of caveolae present on endothelial cells, and by as far as possible using tissues from the mice that can be successfully analysed in the laboratory.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

For animals used in the production of genetically altered mouse models the likely/expected severity of procedures is mild. REDACTED endothelial cell - knockout mice are viable, fertile, have no physical abnormality, and demonstrate healthy blood vessel development with the potential of increased blood vessel formation in certain diseases (Kisanuki et al. Dev Biol 2001;230:230-42; Stahl et al. Blood. 2012;120:2925-9). Caveolin-1-/- mice are viable, fertile and display only mild physical abnormalities within the 3 months they will be used (Martin et al. PLoS One. 2012;7:e46242; Cohen et al. Diabetes. 2004;53:1261-70). Ear notching will be used to mark the animals and this will cause only mild momentary discomfort.

For experiments which require treatment with PI, FITC-albumin and/or dobutamine, all solutions will be prepared aseptically to reduce the risk of infection. Animals will be anaesthetised prior to drug treatments to minimise any discomfort and reduce the risk of error during administration, such as missing the peritoneum and hitting local organs. As animals will be killed via a Schedule 1 method before regaining consciousness, the likely/expected severity of procedures is non-survival.

For animals transferred to the secondary establishment, there is a risk of physiological and social stresses due to the handling, exposure to new sounds and smell, fluctuations in temperature and humidity, disruption of light-dark cycle and separation from cage mates and caretakers. All possible measures to minimise this stress will be taken. These include providing animals will be provided with sufficient space, food and water during transport, and transporting in vehicles with appropriate insulation, temperature control, ventilation and lighting. Once housed at the secondary establishment, animals will be allowed to adapt to their surroundings for at least two weeks to allow them to settle into the new facility.

For animals injected with Matrigel solution, this must occur whilst the Matrigel is ice-cold as it is only in liquid form below 10°C. However, as only a small volume (0.2 ml) is being administered subcutaneously, there are no anticipated adverse effects. Matrigel solution will be prepared aseptically to minimise the risk of infection and will also be administered under general anaesthetic to reduce discomfort to the animal. Animals will then be closely monitored for the next 7-10 days and, if they do exhibit any adverse effects, will be killed humanely via a Schedule 1 method.

At the end of the proposed experiment, all animals will be humanely killed and harvested tissues fully utilised, thus the likely/expected severity is non-recovery.



Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

We have considered using endothelial cell lines to replace mice for some experiments, but our own preliminary experiments in addition to data published by other groups indicate that, while useful for answering specific questions, cell lines do not recapitulate the properties of endothelia present in intact blood vessels in living mammals as they relate to the functions we are studying (angiogenesis = new blood vessel formation; mechanoprotection = limiting damage to the endothelium caused by mechanical stresses such as increased blood flow). However we will as far as possible use freshly prepared and immortalised human endothelial cell lines or other cell types to replace the need for mice as a source of cells and tissues.

Reduction

Explain how you will assure the use of minimum numbers of animals.

The project has been designed to minimise the number of mice needed to make statistically meaningful conclusions from the planned experiments. From our experience we estimate that 10 mice will be sufficient to perform each in vivo experiment. If, through the course of the experiments, we can gain significantly relevant information with fewer animals, study designs will be changed accordingly. This is particularly relevant to the two photon imaging experiments, where data from our collaborators indicate that conclusions can potentially be reliably made using less than 10 mice per condition.

In addition, the increase in cardiac output is to be initiated via dobutamine treatment for consistency and reproducibility, thus reducing result variability. As a result, less mice will be required for statistical analysis than if using an alternative exercise-based method to increase cardiac output.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The mouse is the lowest species known to have caveolae and express REDACTED in the vascular endothelium similar to humans. From a practical standpoint, the abundance of available tools (e.g. REDACTED in which target genes are deleted only in specific cell types) and functional information on both REDACTED/caveolae and REDACTED in mammals coupled with the relative lack of these in lower organisms make mice the only suitable model system for our studies. In addition, several steps will be taken to minimise stress resulting from drug administration (handling methods that minimise stress, single injections, shortest time possible between administration and humane killing) and transport between facilities (2 week period to adapt to new environment prior to express)





145. Spatial Memory in the Hippocampal Formation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Mice	adult, aged, juvenile, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

We aim to understand the general principles of memory formation and consolidation from a neural network level in healthy and neurodegenerative brains.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We are the product of our past; our memories define us. Our daily experience is full of vivid details from various sensory systems; however, our memories tend not to include all of the sensory details. Little is known about how detailed sensory experience is stored into memory, likely through compression and reconstruction for long-term storage. Despite advances in understanding the molecular and synaptic correlates of memory, our neural-level understanding of memory processing is far less mature. I will study information processing for spatial memory in the hippocampal formation in order to gain the mechanistic understanding of memory formation and consolidation.



What outputs do you think you will see at the end of this project?

I intend to publish at least two papers on high-impact academic journals. These publications will contribute to our understanding of how memory works, in particular focusing on the role of the hippocampus and its connections to key areas in the neocortex, with an emphasis on the spatial component of memory.

In the course of my research, I will bring collaborators into my lab, as well as make visits to other labs. This will enable the dissemination of technical skills into the wider scientific community, ensuring that myself and others have access to the most relevant and refined techniques for our research needs. I intend to collaborate both within and outside of my host REDACTED.

I will also nurture and train the next generations of scientists through various means, such as workshops, promoting international collaborations.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The research that my group and I carry out will benefit different fields, including:

Neuroscience and physiology

Clinical evidence suggests that the hippocampus plays an important role in forming our daily memory. Animal studies have shown that several types of cells in the hippocampus respond directly to the location of the animals, which is one of the three key components for memory. My study intends to show the more general and direct contribution of these cells to memory processing in the hippocampus. I will disseminate our results through peer reviewed publications, seminars and conferences, as well as deposit relevant data in open data repositories.

Computational neuroscience

Taking advantage of virtual reality system that we just recently developed, the experiments that I proposed here will take a new angle for understanding the neural basis of memory processing. This detailed data will be valuable to theoretical neuroscientists, and can be used to create increasingly accurate models and hypotheses that can be directly tested in the laboratory. Indeed, a main future goal of my group is to collaborate with the exceptional computational neuroscientists to promote this information exchange.

Brain-machine interfaces

There is growing interest in creating an interface between humans and machines. Such an interface has the potential to return mobility to those with spinal damage and return the facility to communicate to those who have lost it through disease. Through refinement of hardware and data processing techniques, as well as analysis of data, I will push the boundaries of what is achievable in terms of brain-machine interfaces and this will likely be of interest to those seeking to engineer such systems for use in humans.

Dementia



Alzheimer's disease is one of the most common types of dementia, however, effective treatment of the disease is currently missing. Identifying and targeting cognitive deficits at the early stage of the disease is critical for developing effective treatments to reverse or prevent the decline in cognitive functions. By building an understanding of the neural networks involved in memory processing, in particular the part of the networks which is affected early on in the Alzheimer's disease, we will potentially be a step closer to identifying neural targets and novel approaches to tackling the disease.

How will you maximise the outputs of your work?

Many people have an innate desire to understand the world and their own mind. Memory in particular has proven a fascinating subject to the general public. I intend to make my research accessible to the scientific community, as well as the layperson through various way such as conferences, workshops, articles, videos, and in art installations. In some ways it is only through art that scientific knowledge is truly processed on an emotional and intimate level, and where it has its greatest impact on the general population. As such, when I was contacted recently by an artist, I agreed to explore the possibility of dramatizing my work.

In details, to maximize the outputs of my work, I plan the following activities:

All data collected will be made freely available to the neuroscience research community at some point. I will first upload example data with a detailed explanation of data structures to Open Science Framework (OSF.io) as open access when submitting my results to peer-reviewed journals. Relevant data, including modelling data and analysis code, will also be made available through Github as open access, after publication. Finally, all raw data will be made freely available upon request after a suitable period post-publication, allowing exclusive use of the data for research by my own research group and my collaborators.

I will continue my current collaborations with lab within and outside my host institute, and also actively develop new collaborations by organizing workshops and representing my own work in various conferences.

My study is concerned with memory at a network level, and may improve our understanding of the fundamental processes behind dementia, in particular Alzheimer's disease. So, I intend to collaborate with clinicians in the UK, as well as from China, to test the results that I observe in mice to see if we can find analogues in human patients, and potentially look for new therapeutic targets for tackling dementia.

I intend to organize symposiums related to memory processing, using the platforms that are open to the general public to attract clever minds from various backgrounds, bringing in new thoughts from different angles.

In2Science program is organized by a charity and aims to empower underprivileged students to achieve their potential in research careers by providing short-term work placements in various universities. I intend to continue contributing to the program, and encourage my lab members to do so too, supporting and inspiring new generations of scientists.

I will setup my own website and twitter account, giving the general public open access to my research outputs.



I will collaborate with artists and non-scientists, contributing to increasing public awareness and understanding of science. I will be open to consulting on art installations, films, exhibitions, and theatrical performances.

Species and numbers of animals expected to be used

- Mice: 1300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, a wild type or genetically-altered mouse undergo surgical procedures, which result in the attachment or implantation of the assisting devices for neural recording or manipulation.

After the specified recovery period, the animal commences behavioural training. Simultaneously with training and testing of behavioural tasks, the animal undergoes neural recording, with or without neural manipulations, multiple times.

After the completion of the experiment, animals will be humanely killed.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Surgical procedures could potentially have adverse effects for the animals, such as weight loss, pain during surgery, post-surgical complications and infection. Only very small percentage of animals might have one or some of these effects for a short period of time. Appropriate approaches have been described in the protocols to minimize these effects and animals will be humanely killed if these adverse effects continue.

Water or food restriction used for behavioural tasks could potentially have adverse effects for the animals, such as weight loss, dehydration, ill health and discomfort. These effects are expected to be reversed when restriction is lifted. Hence, animals under water or food restriction will be monitored closely and checked daily. Animals will be given full access to water and food if showing any signs of 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 species)?

Around 15% of mice will have a severity level of non-recovery.

Around 15% of mice will have a severity level of mild.

Around 70% of mice will have a severity level of moderate.



What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 study memory processing in the hippocampus at a neural network level. This can only be done in free behaving animals.

In vitro and computational approaches can partially help to understand molecular and cellular basis of the underlying mechanisms of memory processing; however, to achieve the understanding at the neural network level requires the integrity of the network, which can only be obtained by using intact animals.

Our approach of study memory processing is to connect the neural mechanisms to certain behaviours; hence the free moving/behaving animals are required.

What was your strategy for searching for non-animal alternatives?

There are a few non-animal alternatives that we have consider for this project:

Computational modelling;

in vitro studies;

research using human subjects.

Why were they not suitable?

Computational modelling.

We are collaborating/ will collaborate with theoreticians for computational modelling. However, modelling in our fields has it limits due to our limited understanding of the complex neural network. We will use modelling results to validate our empirical data and guide our experimental designs. Ultimately, we will need work on animal to discover the fundamental mechanisms for memory processing.

In vitro studies.

In vitro approaches help to understand molecular basis of the underlying mechanisms of memory processing; however, to achieve the understanding of memory process at a neural network level and establish its connection to behaviours requires the use of intact, free moving animals.

Using human subjects.

Non-invasive imaging methods have been used for studying memory processing in humans. However, these approaches have lower spatial and temporal resolutions,



compared to the in vivo electrophysiological and 2-photon imaging approaches which are mostly available on non-human animals, apart from the limited use in clinic cases. To understand the circuits and cellular basis of memory processing, mice are the least sentient species that we can use.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

NC3Rs' Experimental Design Assistant is used, when applicable, to design experiments and determine the group size, which help to estimate the numbers of animals to be used. In particular, statistical power analysis is used to estimate the number of animals to be used. Pilot study and existing literature are used to estimate important parameters in power analysis.

However, it should be noted that physiological experiments are special in that the number of animals required will largely depend on the success rate of neural recording, which is hard to predict accurately in advance.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Mice are the optimal choice for this project due to the maturity of genetic and imaging techniques uniquely available for the species, and for the rich scientific record on studying memory in freely behaving animals. Specifically, mice are the least sentient species for the study. In line with the Animals Scientific Procedures Act 1986, the project is designed to minimize the total number of animals. NC3Rs' experimental design assistant, when applicable, is used at the experimental design phase for estimating the animal numbers. Based on my previous work, I anticipate that each experiment will require 15-20 data-producing animals in order to achieve sufficient statistical power. However, due to the technically demanding nature of the experiments, I estimate that only 25-50% of animals will produce data. Exact numbers will be adjusted on an experiment-by-experiment basis to minimise animal use.

What other measures apart from good experimental design will you use to minimise numbers?

We will conduct pilot studies with a smaller sample size. This will allow us to test our hypothesis, investigate potential unpredictable effects, examine the feasibility of our approaches and refine our experimental designs and methods, before committing to larger scale experiments involving a larger number of animals.

We will collaborate with theoreticians for computational modelling. We will use the modelling results to validate our empirical data and guide our experimental designs, therefore reducing and minimising the number of animals required.



We will continue experimenting on new techniques, such as higher channel density recording, to maximize the data that we collect for each animal, hence reducing the total number of animals used.

Our recording protocol is chronic recording, which gives us a chance to record different populations of neurons within the same animals. This will maximize the use of individual animals and reduce the number of animals required to draw statistically significant conclusions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice

Main methods:

Behavioural training and testing.

Habituation and behavioural training will be performed to ensure animals to cooperate with recording procedures to minimise any distress. Mild food or water deprivation will be used to motivate the animals. Body weight and other animal behaviours will be monitored and recorded, to ensure the least pain and distress.

In vivo neural recording.

Majority of the data generated from this PPL is from neural recording. There are non-invasive methods of monitoring and imaging neural activity. However, they lack either fine temporal resolution or fine spatial resolution. Most importantly, they cannot provide information about individual neurons.

Therefore, to record neural activity at the single cell level, which is required for studying the questions proposed in the project, it is necessary to implant electrodes for electrophysiological recording or implant glass windows for imaging. The surgical approaches used are the least severe available, involving the smallest amount of tissue damage and the smallest number of minimal-sized holes drilled in the skull. Animals are given extensive post-operative care and closely monitored during the recording experiments and during interactions with other animals.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice have been chosen for the project proposed in this PPL as they are the least sentient species that is appropriate for studying questions regarding spatial memory.



The main focus of the project is to study memory consolidation at a neural network level by investigating the formation of spatial cells and their interactions. The two main classes of spatial cell, place cells and grid cells, were first discovered in rodents and these cells have been heavily studied in rodents ever since. Therefore, mice are one of the most appropriate species for this work as they allow for direct comparisons with past studies. Additionally, the majority of experiments proposed will involve the use of virtual reality techniques that require head-fixing animals to the experimental apparatus, a paradigm which is best-established in mice. Further, several of the other techniques required by the project have been developed specifically for this species, including genetic models, 2-photon imaging, and optogenetics. Indeed, mice are the only species which can take advantage of the combination of all these techniques.

Moreover, less sentient species, for example invertebrates, do not have brain structures homologous to the hippocampus, and thus are not an appropriate model for learning and memory in mammals.

Overall mice are the most appropriate and least sentient species to be used in the project due to the nature of the research questions and the techniques available.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals will be motivated to seek reward using mild food or water deprivation during training and testing of behavioural tasks, we will only use the minimum levels of deprivation necessary to achieve uniform consistent behavioural results (food deprivation to reduce weight to max 85%). Water deprivation will only be used in experiments where food deprivation proves inadequate. An inverse 12 hours day-night cycle is applied to ensure minimal behavioural inhibition, hence to minimize the level of the food/water deprivation required.

The surgical approaches used are the least severe available, involving the smallest amount of tissue damage and the smallest number of minimal-sized holes drilled in the skull.

Animals are given extensive post-operative care including antibiotics and analgesics where they will be deemed to be necessary. Analgesics are always administered pre-operatively.

Animals are closely monitored throughout the experiments and any signs of problems with implants or other aspects of surgery are immediately dealt with, or, if this is not possible, the animal will be humanely killed. Similarly, animals are closely observed and monitored during the recording experiments and during interactions with other animals.

Housing cages will be spacious and enriched with small toys, running wheels, shelters and chewable materials such as wooden blocks, unless these interfere with the experimental design or presented as a potential danger to the surgical implants/attachments. Animals will be housed socially whenever possible (i.e. when no danger to their wellbeing is present as a result, e.g. when another conspecific can cause severe damage to its implant).

For the animals with implants/attachments, extra precautions as follows will be taken to avoid the damage to the implants and ensure the wellbeing of surgicized animals:



Custom-modified cages, in particular, sufficient height of the internal space.

Suitable bedding and nesting materials. In particular, soft tissue is provided before surgery in order for animals to build their nests, which will be kept with surgerized animals.

Food will be placed on the floor of housing cages for easy access.

Selective enrichment which is appropriate, such as running wheels, open-top shelters.

Group-housing whenever possible. However, single-housing may be required when implants/attachments are made from chewable materials or have a significant intruding part.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Resources hosted on the NC3Rs website, in particular:

ARRIVE guidelines on experimental design and reporting results.

NC3Rs' experimental design assistant (EDA).

LASA Guiding principles for preparing for and undertaking aseptic surgery.

'Procedures with Care': 'Aseptic Technique in Rodent Surgery'.

Online general information on:

Rodent housing and husbandry

<https://www.nc3rs.org.uk/3rs-resources/housing-and-husbandry/rodents>

Welfare assessment of laboratory animals

<https://www.nc3rs.org.uk/news/fresh-approach-training-welfare-assessment-laboratory-animals>

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Attend regularly the workshops organized NC3R (National Centre for the Replacement, Refinement and Reduction of Animals in Research) for the updates on advances in the 3Rs.

According to the updates on advances in the 3Rs, I will regularly revisit the protocols in the PPL, consult with NVS and NACWO for their expertise and advice, in order to implement the advances effectively.

Explain the choice of species and the related life stages

Adult and aged wild-type and genetically-altered mice will be used to study the function of the hippocampus in memory processing.



Mice have been chosen for the project proposed in this PPL as they are the least sentient species that is appropriate for studying questions regarding the neural network mechanisms of spatial memory. Histologically, intensive existing studies in mice provide substantial background knowledge and also help to develop a great variety of techniques including virtual reality, genetics and large population neural recording. Choice of mice for this project enables us to take advantage of both existing knowledge and advanced techniques, which are essential for our proposed project.

Both adult and aged mice will be used. Adult stage is chosen to study the function of neural circuits. Aged stage is chosen to understand the biological mechanisms of neurodegenerative diseases such as Alzheimer's disease. By comparing normal to diseased models at the aged stage, we hope to study the pathological anatomical and physiological changes in the diseased brains and connect these with behavioural changes, in order to shine some light on the neural network mechanisms of these diseases.





146. Spinal cord circuits for pain and itch

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Mice	juvenile, adult, 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 is the aim of this project?

Although the spinal cord plays an important role in transmitting and modulating sensory information that is perceived as pain or itch, we still know relatively little about how this information is processed at the spinal level. The aim of this project is to identify and characterize functional populations of nerve cells in the spinal cord that are involved in pain and itch, to establish how they are organised into circuits that increase or decrease these sensations, and to determine how they contribute to pathological pain states.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is a lack of effective treatments for both chronic pain and itch (pruritus), and these therefore represent major unmet clinical needs. Understanding the nerve circuits that convey pain and itch, and characterizing the different cells that are involved, is likely to lead to the identification of potential targets for new treatments for these conditions. In addition, understanding the changes that occur in the spinal cord following peripheral nerve injury is necessary if we are to develop improved treatments for neuropathic pain.



What outputs do you think you will see at the end of this project?

The project will provide new information about the types of nerve cell within the spinal cord, how these are connected to each other to form circuits that process sensory information, and the roles of these cells and circuits in chronic pain and itch. Outputs will include publications in peer-reviewed open access journals.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Chronic pain and chronic itch are both common conditions, affecting around 40% and 25% of the population, respectively. At present, treatment for these conditions is often inadequate, and this is largely a result of our limited understanding of the mechanisms underlying these conditions. The improved knowledge provided by this project should lead in the longer term to the development of new forms of treatment for chronic pain and itch.

How will you maximise the outputs of your work?

We will continue to collaborate with other groups working on spinal cord mechanisms of pain and itch. We will disseminate our knowledge through peer-reviewed open access scientific publications, review articles and book chapters, as well as regular presentation of the data at scientific meetings.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Many of the animals will receive injections into the spinal cord of viruses that alter the expression of genes in specific populations of nerve cell within the spinal cord. These changes in gene expression will result in either an increase or decrease in the activity of the affected nerve cells, and we will test the effects of this on acute pain thresholds and on the amount of itching that results when itch-inducing chemicals are injected into the skin. Acute pain thresholds will be tested by using stimuli from which the animal can readily escape. These sets of behavioural tests will typically be performed on three occasions for each animal. Animals may survive for up to 26 weeks after the injections (in the case of those that are treated as neonates), but the survival time would typically be much shorter (e.g. 6 weeks).

The animals may undergo an operation that results in damage to a nerve in the hindlimb, or they may receive an injection of a chemical into one hindpaw that causes inflammation. Both of these will result in a persistent pain state in which the affected limb becomes more sensitive to mechanical stimulation and to changes in temperature, and again, this will be



assessed by means of similar behavioural tests. In addition, these animals will undergo other behavioural tests, for example to assess the effect of treatments that suppress pain. Again, sets of behavioural test will typically be performed on three occasions for each animal.

Animals will survive for no more than one week after inflammation is induced, and no longer than 8 weeks after nerve injury.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Animals that receive injections into the spinal cord will receive post-operative pain-killers, and should show no more than mild discomfort following the operation. There will be transient post-operative weight loss, but they will rapidly recover and should show no other ill effects.

Animals that receive nerve injuries or develop inflammation will show relatively mild signs of pain that include increased sensitivity to mechanical and thermal stimuli. In the case of inflammation this may last for up to a week, whereas after nerve injury the effects may be permanent. The animals' ability to move around their cages is not reduced after these procedures, and they eat and drink normally.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Most of the animals in this study (~70%) will only be used for breeding and maintenance under Protocol 1, and since these animals will have no behavioural abnormality, this procedure is classified as "mild".

Most of the remaining animals (~30%) will experience moderate severity.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 designed to investigate the organisation and function of nerve circuits in the spinal cord, together with changes in these circuits that underlie chronic pain states, can only be performed on animals. This is because animals provide the only source of spinal cord tissue in which the complex organisation of these circuits is retained and remains functional.

What was your strategy for searching for non-animal alternatives?



Non-animal alternatives would include human spinal cord tissue obtained post mortem or cultured cells.

Why were they not suitable?

It would not be possible to obtain human spinal cord tissue that was sufficiently well preserved for detailed anatomical studies or that retained live neurons and functioning circuits. It is impossible to carry out studies of this type on cultured cells, since these do not have the complex organisation of the intact spinal cord.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Power calculations have been used to determine group sizes for all behavioural experiments, and for other studies where this is appropriate (e.g. for determining the proportions of cells that respond to different types of peripheral stimulus).

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Advice was taken from a local statistician and from online tools for performing power calculations.

What other measures apart from good experimental design will you use to minimise numbers?

Breeding of mice with targeted mutations will involve homozygous animals whenever possible, in order to reduce the numbers that need to be generated to obtain sufficient mice of appropriate genotypes.

Tissue (either perfusion fixed or fresh frozen) obtained from mice will be stored in liquid nitrogen so that it can be re-used for subsequent anatomical 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?



We will use nerve injury models and inflammatory models to investigate chronic pain. These models are all well-established and widely used in pain research, and they have all been refined during the course of previous studies by many laboratories to model clinical conditions. None of these models will cause any significant alteration to the behaviour of the animal, and all animals will be monitored regularly.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice are the lowest animals in the evolutionary tree on which these experiments could be conducted, and are the only possible species that could be used for these studies, due to the availability of genetically modified lines, which allow targeting of different nerve cell populations. Experiments will be performed on adult animals because there are changes in the structure and function of the spinal cord during development. Many of the experiments will be performed on tissue taken from animals that were terminally anaesthetised.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Robust procedures for animal care and monitoring are in place. Environmental and behavioural enrichment are provided. Staff will ensure that all animals receive the highest standard of care. Where appropriate (e.g. before, during or after surgery) preventative measures (e.g. anaesthesia, fluid replacement, pain relief and heated cages) will be used.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will abide by published guidelines (NC3Rs) as well as local guidelines, to ensure best working practice. We will also adhere to the ARRIVE guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs website and elsewhere).

Explain the choice of species and the related life stages

The experiments in this project will all be performed on mice because of the availability of genetically- modified animals that allow specific types of nerve cell to be identified and have their functions altered. Experiments will generally be performed in young adult animals, but in some cases injections have to be given to juvenile or neonatal animals, for example to allow targeting of different populations of nerve cells.





147. Stem cell dynamics during the normal and pathological development of hormonesensitive regenerative tissues

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

tissue development, stem cells, cancer, reproductive organs, tissue regeneration

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to characterise the biological mechanisms that regulate stem cell behaviour during normal and pathological development of the mammary gland. Also, this project aims to conduct preliminary analyses of prostate gland development to ascertain if similar mechanisms exist to regulate stem cell behaviour in another hormonally-regulated regenerative reproductive organ.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 ultimate aim of the outlined work is to advance understanding of the mechanisms that regulate stem and progenitor cell fate and dynamics during mammary gland development and tumourigenesis, and to understand whether similar mechanisms exist to regulate stem cells during prostate development. These studies are important as failures in the regulation of stem and progenitor cell differentiation and dynamics is widely believed to underlie the pathological development of many breast and prostate cancers. In the UK, breast and



prostate cancer are the most common female and male cancers, with around 55,200 and 47,700 new cases diagnosed annually, respectively (Cancer Research UK statistics). The relative five-year survival rate for early stage breast cancer is ~80-90%, falling to ~15% in metastatic disease. Similarly, the relative five-year survival rate for localised prostate cancer is ~100%, falling to ~30% in advanced, metastatic disease. Early diagnosis, therefore, is critical to successful treatment and for maximising the likelihood of survival. Consequently, there is an urgent need to improve strategies for prevention, early detection and treatment of these common cancers.

What outputs do you think you will see at the end of this project?

This project will provide data characterising the dynamics of stem and progenitor cells during normal (including in response to pubertal and pregnancy hormones), and pathological mammary gland development, including tissue injury and tumour development. In addition, this project will provide preliminary data on stem and progenitor cell behaviour and dynamics during prostate gland development. Results from these studies will be disseminated via presentations at international scientific meetings and publications in peer reviewed journals.

Who or what will benefit from these outputs, and how?

Short-term benefits (throughout project):

The data generated by this programme of work will be of value to scientists working to advance understanding of mammary and prostate gland development and pathology, including tissue injury and tumour development. This includes a better understanding of how hormones control the normal development and functions of these tissues, such as during puberty and pregnancy (mammary only), and how they regulate tissue regeneration in response to injury. Those working on the project will gain experience in cutting edge three dimensional imaging and cell tracking techniques, as well as experimental design and data handling. In addition, publication of the findings will help raise the profile of my team and our research.

Medium-term benefits:

By providing new insights into the impact of age, development and reproductive status (e.g. parity) on stem and progenitor cell behaviour during normal development and in response to tissue injury, and the factors influencing their transformation potential, dissemination of study findings will benefit scientist working both in academia and pharma who are focused on identifying improved methods for the prevention, early diagnosis and treatment of mammary and prostate tumours.

Long-term benefits (greater than 5 years):

Ultimately, the findings are expected to contribute to improved public health by the identification of new risk factors and improved strategies for the prevention and treatment of breast and prostate cancers. A better understanding of how reproductive events and their timing (e.g. of puberty and pregnancy) impact cancer risk will help improve patient stratification and cancer early detection.

How will you look to maximise the outputs of this work?



The data generated by the outlined studies will be disseminated via presentations at international scientific meetings, such as the Gordon Research Conference on Mammary Gland Biology and the international ISSCR Stem Cell Meeting, in addition to publications in peer reviewed journals including Cell Stem Cell, Developmental Cell, Development and Nature Cell Biology.

In addition, I will seek to develop collaborations with other scientists working in this research field and other disciplines (e.g. engineering and mathematics), who can use the data generated in our investigations to develop novel approaches to mathematically model stem cell dynamics, or for developing improved image processing techniques.

The novel intravital imaging windows developed in this project, and its scientific and ethical benefits over conventional methods, will be published and publicised at conferences to promote the uptake of this refined methodology.

Species and numbers of animals expected to be used

- Mice: 2915

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 appropriate species and material to conduct research depends on the extent by which the studied process is conserved across evolution and among different developmental stages and tissues. The proposed project relies on the use of transgenic mice to define the mechanisms of stem and progenitor cell regulation in homeostatic conditions, but also during tissue regeneration and in cancer. Among the two tissues that we have chosen as experimental paradigms, the mammary gland and the prostate are specific to mammals, making the mouse the best placed lower organism to conduct our studies. We are interested in the development of these organs, which entails using mice at various developmental stages, from embryo to aged adult.

Typically, what will be done to an animal used in your project?

The majority of genetically altered animals (GAAs) will be administered with substances (e.g. to induce a gene alteration/deletion) and the most common route of administration will be via an injection or through their diet, prior to killing and tissue analysis.

In order to investigate the regenerative potential of cells, some animals will be exposed to radiation and/or undergo drug-induced cell ablation. Some mice, including pregnant mice may undergo intravital imaging, implantation of osmotic minipumps or mammary fat pad clearing which are surgical techniques. The majority of mice, however, will not undergo a surgical procedure.

The majority of experiments will last no longer than 3 months. However, in our pilot studies using GAAs prone to develop mammary tumours, tumours normally arise after 6-9 months. There are no avert adverse phenotypes associated with these GAAs before this time.



In the case of intravital imaging, the majority of these experiments will be <1 month. For irradiation experiments, most animals will be <1 month. Rarely, animals will be kept for longer periods of time after irradiation. In the event of a particular GA having an unexpected phenotype, mice will be taken at earlier time points in accordance to local NVS and Home Office Inspector guidance.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of mice will display no more than sub-threshold to mild clinical signs and will not experience significant weight loss and pain, with no evidence of lasting systemic illness. Some genetic modifications may affect tissues other than the mammary gland and prostate (eg. skin or gut) which carry a risk of some mice experiencing mild to moderate clinical signs (e.g. skin abnormalities, loss of hair, piloerection, weight loss), although it is not always possible to fully predict the nature or severity of any potential defect (e.g. in new GA strains). Thus, for all types of mice there will be careful monitoring for possible side effects. Should these signs persist and cannot be ameliorated by mild veterinary intervention, they will be killed. Some mice will be subjected to higher doses of irradiation which carry a risk of radiation-induced weight loss and a compromised immune system. Animals will either be euthanised prior to appearance of these clinical signs or for longer term studies receive an injection of haematopoietic stem cells within 24 h of radiation to enable their recovery and prevent the appearance of these clinical signs.

Animals undergoing a surgical procedure, including pregnant animals (e.g. implantation of intravital imaging, osmotic minipumps, fat pad clearing) may experience transient moderate clinical signs after surgery. These will be controlled by an appropriate post-op analgesic programme and the majority of animals are expected to make a rapid and unremarkable recovery from the described surgical techniques. Implantation of imaging windows during pregnancy carries a small risk (<5%) of abortion or birthing difficulties.

Some animals will develop mammary tumours. These are external tumours that will not be allowed to grow to diameters exceeding 1.2cm. Thus, the vast majority of mice bearing tumours will not show any overt adverse effects as these tumours will not affect any vital function or have any systemic effects as they will be highly unlikely to have metastasised to internal organs by study endpoints in the chosen mammary tumour models.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

From total animal numbers:

Mild and sub-threshold in >90% of cases.

Approximately 10% may experience moderate clinical signs, which will be dependent on the nature of the genetic alteration and response to cell ablation/injury. <5% of animals may undergo a surgical procedure, which carries a moderate severity. However, the majority of animals will make a rapid and unremarkable recovery from the described surgical procedures. <2% of animals may develop mammary tumours which may mean that a subset of these will experience clinical signs of a moderate severity, and these animals will be killed if they cannot be controlled by minor veterinary interventions.



However the vast majority of the <2% that may develop mammary tumours will not show any overt adverse effects as these are external tumours and experiments will be terminated before metastasis 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?

The choice of appropriate species and material to conduct research depends on the extent by which the studied process is conserved across evolution and among different developmental stages and tissues. The proposed project relies on the use of transgenic mice to define the mechanisms of stem cell regulation during development and homeostasis of the mammary gland and prostate in physiological conditions. By its very definition, the mammary gland is specific to mammals, making the mouse the best-placed lower organism to conduct our studies. Similarly, the mouse is currently the best available system for studying prostate development. In addition, transgenic mice currently represent the best model system for studying cancer and its biological basis. Notwithstanding our efforts towards alternative methods that replace the use of animals, namely the use of organotypic cultures to model organogenesis and stem cell behaviour *in vitro*, we will critically require to perform some experiments in mice, in order to visualise mammary gland and prostate development in their natural and physiological microenvironment. The rodent model is the least severe approach to addressing our objectives, and are therefore necessary to complete the goals of the proposed research.

Which non-animal alternatives did you consider for use in this project?

Where appropriate we will use *in vitro* models of immortalised primary cell lines and 3D organoid cultures to undertake more detailed functional and molecular analyses of individual pathways and genes, in addition for screening chemical compounds and/or growth factors that are considered candidate effectors of mammary and prostate stem cell behaviours. Time-lapse imaging of 3D organoid cultures will also be used where appropriate (e.g. when we don't require the presence of other cell types in the microenvironment) to support the proposed intravital imaging experiments, which will ensure that fewer mice undergo this technique.

My lab is also focused on undertaking genetic epidemiology analyses of human populations to investigate the causal impact of developmental exposures on breast and prostate cancer risk. Collaborations with genetic epidemiologists are underway, which are generating hypotheses that can be tested in the laboratory using human primary cells and tissues (obtained from tissue banks). This will reduce the number of mice used during this project, as we will bypass mouse models completely to investigate promising pathways and targets identified by genetic epidemiology approaches in human tissues only.

Why were they not suitable?



The mammary and prostate glands are complex tissues comprised of multiple cell types that undergo phases of rapid growth and regeneration. It is not possible to mimic this completely in culture. *In vitro* models do not recapitulate the complex physiology of tissue and disease development, as they do not include the full spectrum of different cells that may a role in these processes. Particularly, current *in vitro* techniques cannot model the complex and fluctuating systemic hormonal environment crucial for regulating prostate and mammary gland development and tumourigenesis. However, I have developed a 3-dimensional organoid culture model of the mammary gland that can be used to replace some of the animals that would otherwise be required in this project. In addition we will rely on human primary cells and tissue from available resources (e.g. tissue banks) wherever possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This project will require complex genetic crosses. We will design the mating protocols to maximise the number of mice with the correct genotype and will keep the number of mice as low as possible in initial crosses until the desired genetic combinations are achieved. However, some strains may be unable to be maintained as homozygotes, meaning that these breeding strategies are less efficient than with homozygous strains. Thus, breeding is estimated at approximately 400 animals per year based on my previous experience of complex genetic crosses encompassing a number of alleles. By including a pilot study investigating prostate development, we will use some male mice during this project, thus decreasing the numbers of animals that will be killed due to not being the correct sex for the majority of experiments. Mice of the incorrect genotype will be used as controls, replacement breeders or in experiments used to develop our new intravital imaging windows, which will not be as heavily reliant on having all the alleles required to address the other objectives of the project.

Experimental usage is estimated at ~150-200 per year, which is around 10 experiments per year divided between 2 researchers, based on an average effect size Cohen's d of between 1 - 1.4 (using a dependent or independent t-test as required) with power of ~80% (0.815) and a probability (α) of 0.05 for the majority of experiments. Detailed justification of mouse numbers is provided within each protocol. However, as our group grows and more funding is acquired we may perform experiments in different strains (e.g. different tumour models, new GA mice based on results of RNA-seq experiments etc), which will mean that these numbers will need to be adjusted accordingly.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In addition to seeking the advice of a local statistician, online resources were consulted including the NC3Rs' Experimental Design Assistant and online tools such as G*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 throughout and pilot studies will be performed where appropriate to ensure that the minimal numbers of animals are used during the course of this project. Tissue sharing occurs on an ad hoc basis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use the mouse as our model during the course of this project. Our work requires modulation of specific genes and the mouse is the only model that we can use to carry out genetic studies in the mammary gland, and the best placed lower organism for studies involving the prostate. Inducible conditional gene targeting will be used to delete the gene under study only in the mammary gland or prostate wherever possible. This will provide better experimental data, in addition to reducing any adverse effects from deleting the gene in other tissues, thus representing a refinement.

Most of the protocols are in the mild category and animal suffering will be minimal. For longitudinal intravital imaging studies, only animals destined for imaging will have the intravital imaging window implanted, and mice will be killed immediately if images of sufficient quality cannot be obtained.

In mammary gland tumour studies, the size of tumours may be up to 1.2 cm at the widest point. Postmortem assessment of tumour growth in our pilot studies, however, will be used to refine end points further.

Why can't you use animals that are less sentient?

Mammary gland and prostate development and tumourigenesis occurs postnatally. Thus, we will perform most of our studies in animals older than 21 days old. However, some studies (e.g. regarding the role of stem/progenitor cells in the first branching event in these organs) will be performed in embryos where appropriate. For studies involving long-term time-lapse intravital imaging, animals will not be allowed to recover and will be killed at the end using a schedule 1 method while still under anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Pilot studies will be performed to refine doses of radiation, administered substances and tumour endpoint sizes, which will also provide valuable statistical data that can be used to refine cohort sizes in future experiments. Post-mortem assessment of tumour growth in our pilot studies will also be used to refine end points further.

We will continue to refine our methods for monitoring animal discomfort after surgeries and clinical disease in tumour models. This includes an analgesic-anaesthesia management



plan in accordance with the local named veterinary surgeon (NVS). We will use general anaesthesia and administration of analgesics to all procedures considered more traumatic for the animal than the introduction of a needle. This includes cases where only very small incision sites (<1cm) will be performed. We use a general gaseous isoflurane anaesthesia carried by a mixture of oxygen and compressed air. When this is not possible, we use a general anaesthesia by injection, using a mixture of xylazine/ketamine/flunitrazepam as per NVS guidelines. During the time of the procedure requiring anaesthesia, mice will be kept on a heating pad in order to maintain the temperature of its body. After surgeries, animals will be maintained under a infrared lamp until they wake up. Continued analgesics will be provided in days after surgeries where required and in accordance with the NVS recommendations. Specialised bedding and enrichment will be provided to animals undergoing any surgical procedures.

All animals will be assessed daily for signs of adverse effects. However, monitoring regimes and frequency will be adapted as necessary to be proportionate to anticipated/observed effects, e.g. use of scoresheets if adverse effects are noted and when mice possess mammary tumours. All mice will be monitored in accordance with the NC3Rs Welfare Assessment Guidelines.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will participate actively in 3Rs seminars, presenting our approaches and learning from others.

Mammary gland related protocols are published as methods books, which are often updated to include more recent techniques and refinements. The more recent edition was published in 2017 and we will refer to these publications where relevant. In addition, researchers are increasingly publishing refined protocols as videos online (e.g. JOVE) which we will also use to keep ourselves updated. In addition, I routinely attend the annual Mammary gland methods workshop run by the European Network of Breast Development and Cancer Labs (of which I am a member), where recent technical advances in the field are publicised, including refinements to standard mammary gland specific protocols.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By continually keeping up to date using resources online, following related accounts on Twitter and LinkedIn and by participating in local, national and international meetings focused on animal research.



148. Stock Centre and Cryopreservation Programme

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Zebra fish	embryo, juvenile, adult
Killifish	embryo, 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 is the aim of this project?

The purpose of this project licence is to create a centralised stock centre service to efficiently breed and maintain a source of fish, reproductive and genetic material for use in scientific experiments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 benefits of this aim are threefold: reduction of the number of fish being used within the facility; refinement of current husbandry methods; acquisition of highly technical skills.

A centralised stock centre is an efficient method for producing zebrafish lines, allowing reduced numbers of fish to be shared by multiple users. This principle has already been demonstrated in the current stock centre programme: multiple PiLs, under several different PPLs, are sharing popular lines, which has reduced the numbers of overall fish. It is also the principle employed by Zebrafish

International Resource Centre and European Zebrafish Resource Centre. By offering this as a service, to both on-site users and off-site users, we reduce the total number of fish required for scientific use. Many experiments use embryonic forms which are under 5 days



post fertilisation, and the centralised service allows us to use fewer adult fish. Its use is a refinement of colony management as it allows for a reduction of total animals used; as all the users effectively share the same fish, there is no replication of lines. The stock centre is maintained by highly skilled staff, who specialise in the maintenance of the available stock.

What outputs do you think you will see at the end of this project?

This is a service that will see a reduction in total number of animals used, and a rigorous form of colony management through the use of cryopreservation.

Through this work the facility also aims to develop refinements to breeding practice and programmes and which are o benefit to animal welfare. We always disseminate this information by presentation and publication to other interested stakeholders including researchers, veterinarians, legislators and other technical staff.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

This will reduce the numbers of fish used, because genetically identical animals will not be reproduced across different PPLs. The service will also provide zebrafish to other scientifically approved establishments.

The cryopreservation programme will ensure the minimum numbers of fish will be held alive, while ensuring that all relevant genetic material is still available.

Additionally, the work we do in the stock centre will also benefit researchers, technical fish facility staff and external communities, if they chose to take up our ideas about colony management.

How will you maximise the outputs of your work?

These services are widely advertised with in the facility, with internal users free to brow the lines held on our database. We will look to advertise them more broadly outside of our own establishment, including adding a database to our webpage showing lines available for bona fide scientific establishments to browse and order from.

We actively disseminate developments and refinements we make to breeding practices by actively engaging with stakeholders through presentations at conferences, publication and public outreach. Our presentations and publications are to targeted audiences – for example we present developments to animal technologists in a different way than we might to researchers, reflecting their different needs.

Species and numbers of animals expected to be used

- Zebra fish: 20,000
- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Protocol 1 describes the breeding and maintenance of fish lines, for the most part this is natural spawning. In some cases, these fish may be genotyping using a biopsy of the caudal fin or muccal swab. Protocol 2 describes the cryopreservation programme – which employs both cryopreservation and in vitro fertilisation. It is likely that most animals on this project licence will only be used for natural spawning, with a small percentage undergoing genotyping using a biopsy of the caudal fin, as most lines will be genotyped using fluorescent imaging of embryos under the age of 5 days post fertilisation.

Fish used in the cryopreservation programme will have potentially undergone caudal fin biopsy or muccal swab to determine genotype. These fish will be held briefly under general anaesthetic while gentle pressure is applied to the abdomen to extract gametes up to a maximum of 5 times with a rest period of at least 2 weeks between each gamete extraction. The majority of fish in the cryopreservation programme are unlikely to experience gamete extraction 5 times – a more likely experience would be 2 gamete extractions with a 4-week rest period in between.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

There are no expected adverse effects from natural breeding.

Potential harms may arise from the use of anaesthesia, which would include gill bleeding and this may happen in both anaesthesia in the cryopreservation programme and in genotyping using the caudal fin biopsy method.

Bruising from applied pressure may occur in the abdomen from the cryopreservation protocol, which can impede swimming.

In very rare cases, infection may occur from breaching the mucus membrane when taking a muccal 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 species)?

Both protocols are mild and all fish in the programme are expected to fall within either the mild severity limit or into subthreshold.

In the breeding protocol, it is expected that 5% or less will fall into the mild category with the other animals falling into subthreshold category.

In the cryopreservation protocol, all animals are expected to fall in the mild severity threshold.

What will happen to the animals at the end of the study?



- 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?

Fish are an increasingly popular research model and our aim is to provide a service to those using fish in scientific research setting for experimental purposes. Because of the nature of this project licence it is not possible to choose a replacement to fish.

What was your strategy for searching for non-animal alternatives?

None

Why were they not suitable?

We require animals to provide the necessary service.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 animals we need to use is based on animal numbers used in the REDACTED, including amendments to the PPL to reduce the numbers of fish needed due both to changing scientific need and to the trials within the facility to refine and make of practices more efficient.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

As a service PPL, the main purpose of this project licence is to consolidate fish line sharing between research groups both within and externally to the establishment and reduce numbers of fish used in other project licences overall.

We constantly check and monitor other project ensure that there is no duplication of lines of fish at the establishment, I attend a monthly fish facility strategy board meeting, at which colony management is regularly discussed. We have also designed a colony management taught course to help people manage colonies and we regularly monitor individual's fish via the database. We have also been instrumental in introducing our research groups to the NC3RS onsite representative and regularly setup legislative refresher courses for our research groups to remind them of their legislative obligations and the use of the 3RS. All of which is designed to address poor experimental design in the projects we supply fish to.



As well as using data harvested from the previous stock centre licence, we also calculated the numbers of fish required for the project, we used both the NC3Rs experimental design calculator and consulted with statisticians both internal to the establishment and external.

What other measures apart from good experimental design will you use to minimise numbers?

We use efficient breeding and considered colony management. We have setup many trials over the years, with the aim of increasing good welfare practice and reducing the numbers of fish used in research both at the establishment and more generally in the field. For this work and to help calculate the numbers of fish in the stock centre, we have used both the NC3Rs experimental design calculator and consulted with statisticians both internal and external

We regularly attend conferences and present this work. I have extensive networks and assess to different groups which allows us to keep up to date with new practice with in the field internationally.

We also actively promote the stock centre and cryopreservation service as we believe the sharing of animals between several other PPLs ensures optimal use of the animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

This is a service licence and the goal is to reduce the amount of fish produced across several other project licences, other establishments in the UK and other bona fide establishments worldwide. We will do this in two ways by identifying popular lines of fish through working group meetings that include researchers and by database analysis and then combine We will supply wildtype embryos and wild type adults for various purposes – including crossing to other strains of fish. We will also hold genetically altered lines of fish that are popular with researchers working on different project lines to consolidate lines, so we do not have duplications of lines within the facility, these are distributed as embryonic forms to interested scientific researchers be stored for future derivation and are not held. A cryopreservation service is also offered, this allows lines of fish gametes to be frozen and stored when lines of fish are not actively being used – but may be required for further future scientific use. This is a refinement because it means that live fish are not being used.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

This is a service exclusively for zebrafish, killifish, Cavefish and a small colony of wild type Giant Danio, and as such it is impossible to use an animal that is less sentient.



What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We will constantly review this project licence, which is possible through information mined from our extensive database, containing records going back almost 20 years and information about the fish lines including colony management, survival rates, breeding success, health status and cryopreservation success. We also regularly review the literature, attend conferences and workshops to ensure that all procedures we perform are as refined as possible.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

I follow Home Office guidance, but also follow other sources and publications including Zebrafish, Animal Welfare Technology, other publications. I perform publication review and speak to a wide variety of colleagues including legislators, research scientists, animal technologists and my extensive peer group.

For example –

Guidance on the operation of the Animals (scientific procedures) Act 1986 - <https://www.gov.uk/guidance/guidance-on-the-operation-of-the-animals-scientific-procedures-act-1986>

Zebrafish - <https://www.liebertpub.com/loi/zeb>

Animal Welfare Technology - <https://www.iat.org.uk/atw>

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I have very varied contacts with in the community, which include the NC3Rs, IAT and LASA and the Home office as well as constant contact with my peer group. All advances and new ideas will be assessed and implemented if applicable.

Explain the choice of species and the related life stages

These fish are part of a husbandry and welfare service to supply highly popular lines to multiple research groups, who would otherwise generate many more fish by individual research group duplication. We also hold lines for research groups in preparation for the fish to move onto other project licences at points when this is impossible in the first place, for example researchers who are newly arrived at the establishment and are still awaiting their own project licences. The cryopreservation service allows the archiving and re-derivation of lines with a useful scientific need and reduces the number of live fish. We determine the life stage of the fish generated either by the requests of researchers or, in the case of the cryopreservation programme by the demands of the technique.



149. Studies of Neointimal Formation in Response to Vascular Injury

Project duration

5 years 0 months

Project purpose

None selected

Key words

Heart attack, Vascular graft, Stenting

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

Heart attacks cause more deaths in the UK than any other disease and survivors of a heart attack have a marked reduced life-span and quality of life. Heart attacks are most commonly caused by a disease called atherosclerosis, which involves the formation of a blockage within the hearts blood vessels (coronary arteries) which stops blood flow. If a patient survives a heart attack, the current treatments for blocked coronary arteries are usually to either bypass the blocked artery with a section of the patients vein taken from their leg, or to reopen the blocked artery using a medical device called an angioplasty balloon which permits the placement of a wire-meshed tube (called a stent) to hold the artery open and allow blood flow. These treatments save many lives, but both have high failure rates in the long term as they cause injury to the vessels, causing them to re-block. These processes are called vein graft failure and restenosis respectively and are triggered by cells (vascular smooth muscle cells) from within the blood vessel moving and growing inwards to form a new blockage, termed neointimal growth and formation. As such, learning about how these blood vessel cells are regulated will provide useful information for the development of new medicines for the treatment of these underlying causes of such cardiovascular 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.

What are the potential benefits that will derive from this project?



This project will advance our understanding about how blood vessel cells and their associated molecules are involved in normal artery and vein function and how they are altered to contribute to re- blockage of bypass grafts or stented arteries, and ultimately the formation of new blockages that require further surgery or cause sudden death. If we can find which pathways or molecules cause the vessels to re-block and find a drug to prevent this from happening, then work within this project in the mid-to- long term may lead to the development of safe and more effective therapies. This would reduce the need for patients to undergo another angioplasty and stent deployment or further bypass surgery, and even prevent death from a second heart attack. Instead they may be able to take a pill to prevent such occurrences or it may be possible to treat the vein graft with a drug before it is used or add drugs to the stent, to improve their viability and applicability.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

This project proposes to use mice. Approximately 1500 mice will be used over 5 years (300 mice per year).

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Approximately 50% of mice will be maintained for breeding purposes and to provide animals for neointimal formation experiments, and this procedure is therefore classified as mild as no adverse effects are expected. Approximately 50% of animals will undergo surgery (15-20 minutes) in order to tie- off one of the carotid arteries or have a substance applied to the outside of the artery, in order to induce neointimal formation. At the same time some of these mice (about 50% of mice having surgery) will have osmotic mini-pumps implanted to deliver drugs/molecules under therapeutic investigation. In both instances, mice are expected to recover quickly and will be given pain killers and post-operative care just like people recovering in hospital. Due to the nature of the surgery this protocol is considered moderate, although no serious adverse effects are anticipated as our previous studies have shown that mice recover well following surgery and are feeding and behaving normally a few hours after their surgery. However, induction of neointimal formation within coronary arteries may result in sudden death due to a heart attack in a small number of mice (less than 5%). Animals will be killed at the end of experiments by an approved Home Office humane method and blood, tissues and cells taken for further laboratory study and scientific analysis after death.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Laboratory studies using isolated cells and tissues from either mice or humans will form a major part of the proposed studies and will be used alongside our ex vivo organ culture



system to determine potential interventions in advance of any work in live animals. However, the complex biochemical changes that occur in response to vascular injury cannot all be modelled in isolated cells or ex vivo tissues, because they are influenced by a wide range of physiological factors that are unique to living animals. The study of whole tissues or cells isolated from those tissues facilitates refinement of our studies, as certain interventions and agents can be used that would not be possible (for reasons of toxicity, rarity or cost) in living animals. It also enables us to reduce our use of animals because many cells can be isolated from a single tissue and used for multiple studies.

Reduction

Explain how you will assure the use of minimum numbers of animals.

The number of animals will be minimised by conducting initial studies in cells and tissues in the laboratory, with strictly controlled conditions to minimise experimental variability. Our extensive experience of such studies means that we can use historical data to perform power calculations to ensure that the experimental designs are biologically and statistically rigorous. In general, the experimental design will involve comparison of a control group with one or more intervention groups using statistical tests appropriate to the data. We will regularly review our designs in the light of the data generated to ensure that the results are statistically rigorous but do not involve the use of unnecessarily large groups of animals.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The vascular injury mouse models we have selected are virtually unique amongst experimental animals in that they develop neointimal formation (the root cause of secondary heart attacks in humans) without the need for intra-vascular injury and associated prolonged anaesthetic, so we have chosen them for our studies of the biological basis of vein graft failure and in-stent restenosis. Mice are also an appropriate species due to the availability of genetically modified strains and active inhibitors. Moreover, we have refined our use of osmotic mini-pumps to ensure we use the smallest models available, further limiting any potential adverse effects associated with their deployment. We have extensive experience in all of the models and methods to be used in this project and are confident that they are the most appropriate to address our research questions.

We will always use the least invasive procedure alongside anaesthetic and analgesic in such procedures to minimise animal suffering. We will also continuously monitor the outcome of our procedures in order to effectively minimise this suffering.



150. Studies of retinal ganglion cell damage in experimental glaucoma and optic 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

No answer provided

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

Glaucoma is a major cause of vision loss. It is caused by the loss of neurons that transmit visual signals from the eye to the brain. Using a rat model of glaucoma we determine whether it is possible to restore these connections and restore vision that has been lost by the time a patient is diagnosed. These treatments are likely to be drugs that are injected into the eye (a common route) to enhance the ability of remaining neurons to self repair.

The aims of the project can be summarised as:

1. Quantification of the extent to which retinal neurons degenerate in glaucoma.
- 2 The extent to which these cells can be recovered following treatment.

The objectives are:

The development of a treatment regime for the recovery of retinal structure and function in glaucoma

The development of techniques to show, definitively, that retinal recovery has occurred.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.



What are the potential benefits that will derive from this project?

Glaucoma remains one of the major causes of vision loss in glaucoma. While there are effective treatments for many patients – usually by reducing the eye pressure, significant numbers of patients still experience significant vision loss. Current treatments focus on the prevention of progressive vision loss. No treatment has yet been developed for the recovery of vision that has been lost. We will address this shortfall by studying the earliest degenerative events the cells that transmit visual signal from the eye to the brain (retinal ganglion cells). In glaucoma, these cells lose their connections with the retina and can no longer transmit visual signals to the brain. Since they retain their connections with the brain they form the basis for developing treatments to restore the circuit that takes visual information from the real world to the brain. In these experiments we will determine the extent to which these connections and circuits can be repaired following the injection of drug into the eye. The ultimate goal is that we can restore vision that has been lost in glaucoma.

Another key benefit is that we will know the extent to which these drugs can repair the retina. This information is essential for the next step in developing a new treatment - a clinical trial. Results from work in our models of glaucoma will give some idea of the doses of drugs to be used and the size of the beneficial effect, information which is essential for knowing how many patients should be recruited to a trial and the nature of the trial end points (i.e. when we might say that it has or has not been successful).

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Rats, adult: 200; Mice, adult 650; Over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The maximum anticipated adverse effects are moderate. We induce mild damage to one eye in each animal and this is to model an eye condition that is (in terms of discomfort) asymptomatic. The majority of adverse effects relate to drying of the eye during examination or mild reactions to applied medications. Animals could experience a corneal abrasion- but these usually recover within 6 hours and are very rare (no cases to date in our group).

For the bead model of glaucoma induction, the degree of discomfort is minimal: animals show no change in behaviour following treatment. With the optic nerve crush model the effects on animal behaviour are minimal since we only aim for partial optic nerve crush and therefore ensure that the treated eye has residual vision. The possible adverse effects are that infection could occur at the site of ocular injection or conjunctival manipulation. These have not been issues with these techniques in our hands.



We will use LPS to activate the innate immune system but at low levels that are below the threshold to induce uveitis or significant weight loss. The greatest effect is that the animal can lose up to 20% of body weight- but our experience with this technique is that the weight loss is c 10% and animals recover in the weeks following injection.

All animals will be killed at the end of the protocol. Any animal that exceeds the threshold level for adverse effect will be killed.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Demonstration that retinal ganglion cells in experimental glaucoma requires an intact visual pathway. We need to demonstrate the anticipated route of delivery (by intravitreal injection) is effective and that functionally retinal ganglion cell show improvement over a 2-3 week period. Mice and rats have sufficient similarity with the human visual system in terms of optic nerve anatomy to justify their use.

Reduction

Explain how you will assure the use of minimum numbers of animals.

We can perform dose ranging studies using retinal explants. We can also ensure high cellular yields by using multiple coloured dye labelling to label multiple retinal ganglion cells in each eye.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Both mice and rats have well developed optic nerves with features that match structure seen in human.

The method that we have developed to increase intraocular pressure allows the controlled elevation in IOP in 90% of animal injected. Our IOP increases are modest and can be mitigated by the application of oculohypotensive medication. Both rat and mice tolerate the elevation of IOP well with no observable effects on activity/ weight.





151. Study of Haematopoietic stem cell gene therapies to treat genetic-associated 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

No answer provided

Animal types	Life stages
Mice	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

To assess the function and efficiency of blood (haematopoietic) stem-cell based gene therapeutic approaches to correct inherited genetic disorders and novel disease targets

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Using blood stem cells, that are responsible for producing other cells of the immune system, for stem cell gene therapy, is emerging as a successful approach to treat several inherited genetic disorders. This involves making a functional copy of the gene in the laboratory and inserting it into a sample of the patient's own blood or bone marrow stem cells, using a modified virus so that the patient's defective cells now carry the correct genetic information. These genetically modified stem cells are then given back to the patient as an autologous (using one's own cells) stem cell transplant, where they can continue to develop into corrected blood cells.



Developments from both academic and biotech sectors have driven this approach through to clinical use, and stem cell gene therapy is now proving to be a highly successful, safe and long-term transformative treatment for patients with several life-limiting inherited disorders (including X-linked Chronic Granulomatous Disease, Wiscott Aldrich Syndrome, Metachromatic Leukodystrophy, Beta- Thalassemia, ADA-Severe Combined Immunodeficiency). Despite this progress, there is an urgent need to improve the processes for the manufacturing of cell and gene therapies to i) improve the drug product, delivering a higher chance of clinical success and therapeutic benefit to patients, and ii) to reduce the costs of manufacturing gene cell therapy products, as this currently presents the major barrier to making it more deliverable and accessible to patients.

In addition, founded on the progress and emerging clinical data achieved by current stem cell-based gene therapies, it is also now important to explore whether this approach can be used to treat other immune based inflammatory disorders, for which only palliative or prophylactic treatments are available. Basic research into whether a stem-cell based gene therapy approach can provide an effective and long-term treatment for these disorders is currently lacking. Demonstration of the safety and successful use of stem cell-based gene therapy by animal experimentation is an important component of this translational research programme and is a prerequisite for further research to develop advanced therapy investigational medicinal products.

This research will therefore address a clear healthcare need for several inherited diseases and chronic clinical indications. Firstly, it will provide key data to inform direct translation of the project findings into our current process for development and manufacture of clinical drug products. Secondly, it will provide proof of concept data for the application of this therapeutic approach to treat novel disease targets with limited treatment options.

What outputs do you think you will see at the end of this project?

The resulting data from this Project will be published in peer-reviewed scientific journals and presented at national and international research meetings. Findings relating to improved stem cell gene therapy will be transferred and implemented into clinical drug product manufacture.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

We expect to make significant advances in the development and safety testing of novel cellular therapies for rare inherited disease and other diseases associated with known monogenetic defects.

The optimisation of methods that result in better gene modification will significantly improve our current processes to manufacture a gene modified stem cell medicinal product. Successful use of these modifications to the current manufacturing processes will improve the therapeutic potency of the stem cell product and dramatically reduce the cost of this therapy, making it more affordable and therefore more accessible to more patients in need. As the experiments designed in this Project will use cells and materials prepared to similar specifications as clinical therapeutic HSC-gene modified drug products, the results will be readily transferable to clinical manufacture. As such the developments investigated may be translated to our cell therapy manufacture process within 2 years of completing this research.



Validation that stem cell gene therapy may be a successful approach to treat highly prevalent chronic diseases such as irritable bowel disease, will lead to continued investment in further research programs for pre-clinical and clinical evaluation.

The wider impact of successful project outcome and implementation could ultimately contribute to

Development of novel therapeutic approaches to chronic inflammatory disorders for which there are currently no long-term treatments available

faster patient access to life-saving and life-changing therapies at more affordable prices

reduced treatment costs and financial burden for payers (NHS, tax payer)

How will you maximise the outputs of your work?

Although the results of this research ultimately aim to translate the findings to clinical application, all data generated, including unsuccessful approaches for stem cell selection and enhancement of gene modification, will be published. This will ensure the outputs of this Project are maximised.

Species and numbers of animals expected to be used

- Mice: 1300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mice may be weighed and experience regular (e.g. weekly) blood sampling (e.g. via tail vein nicking). Mice will receive a non-myeloablative conditioning treatment of either whole body irradiation, or up to 4 intraperitoneal injections of chemical conditioning agents administered over 4 days. Mice will then receive a graft of cells via a single intravenous injection. Mice may experience non-invasive imaging under anaesthesia after receiving a substrate tracer injection via intravenous route. Mice will be maintained for up to 6 months for study and then be killed by schedule 1 and non-schedule-1 methods.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Adverse effects that mice may experience include:

Transient and acute pain from injections

Acute Peritonitis as a result of intraperitoneal injections
chronic xenogeneic Graft-versus-host disease as a result of transfer of human cells



Up to 20% weight loss and loss of body condition as a result of myeloablation or combined impact of adverse events of protocol steps

Use of humane endpoints will limit any other potential adverse effects or any clinical symptoms as a result of the procedures to be performed

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Mice within this Project will experience procedures with Mild or Moderate Severity. The proportions of the total mice to be used in this study that will experience each are:

Mild: up to 5% of mice

Moderate: up to 95% of mice

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 in vitro assays as much as possible to replace in vivo experiments. However, while in vitro assays provide insight into the stem cell multipotency or, the function of the therapeutic protein they are genetically modified to express, these assays cannot accurately model the multiplicity of immunological factors or networks of interactions that occur in vivo, which determine engraftment potential or functional gene expression in a physiological setting. The current gold standard assay to experimentally test the repopulating potential of human HSCs is intravenous or intra-bone injection into sublethally irradiated, genetically immune-deficient mice.

Furthermore, in order to fully understand interactions between the immune system and tissues affected by diseases related to dysregulated immunity, we need to use transgenic/genetically altered mice bearing alterations in disease-causing genes. The use of in vivo models is essential to refine existing (and develop new) cellular therapies as they allow us to detect in vivo homing and persistence of transferred cells, their efficacy in modifying/preventing disease and off target toxicities.

We will abide by the recommendations laid out in the following guidelines: (i) Refining procedures for the administration of substances [www.lasa.co.uk] and (ii) Guidance on the use of human material in animals [<https://www.gov.uk/government/publications/guidance-on-the-use-of-human-material-in-animals>]

What was your strategy for searching for non-animal alternatives?



We will endeavour to use in vitro assays wherever possible to characterise and study the function of human stem cells. Although some study's use in vitro 3d culture systems to evaluate human embryonic stem cell and induced pluripotent stem cell function, these do not provide information on long-term engraftment potential and have not yet been successfully applied to primary human HSC stem cell research. We will use LTC-IC assays (long-term culture initiating cell) and CFC (colony-forming cell) assays to detect HSC early and late progenitors, to inform the planned in vivo assays.

In addition, where possible, primary clinical material and human cell lines have been sourced to conduct parallel work, to support and inform study components requiring animals.

To evaluate the impact of specific gene defects associated with disease indications, we will generate knock out human and murine cell lines for initial testing in vitro, to formulate clearer hypotheses and inform in vivo experimental plans.

Why were they not suitable?

Other than ex vivo and in vitro cellular assays, no replacements are available to assess or accurately predict human stem cell ability to migrate to the bone marrow and survive once transferred into a living organism. The humanised mouse protocols developed to study haematopoietic gene-modified stem cell function have been selected as they allow the engraftment of human stem cells, as previously demonstrated by ourselves and others. Combined with the successful engraftment of human cells and reconstitution of a functional human immune compartment, the humanised mouse model and transgenic mouse models provide an important bridge to study the therapeutic benefits or side-effects of the gene being transferred and expressed in an experimental disease model.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The sizes of experimental groups and the number of repeated experiments will be kept to a minimum while ensuring that reproducible results are obtained with clear biological significance, ensuring comparable levels of data can be achieved while using fewer animals. From my previous experience using humanised mouse models described in Protocol 2, and murine bone marrow transplants in Protocol 3, the methods have been refined to maximise the number of mice that engraft with a human immune system, and to optimise the degree of engraftment and minimise the numbers of mice lost due to technical failures.

When preliminary data is available from our and others published research experience of these in vivo models, power analyses will be used to determine the minimum numbers of animals and repeated experiments that are required to meet statistical significance. All such calculations will be made in consultation with the departmental statistician.



What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Selection of experiments to be performed in the Project have been informed by in vitro data, to minimise the number of mice and experimental conditions required. For example, in Protocol 2 of this Project, initial conditions tested in vitro included evaluating the function of 8 HSC stem cell subsets (Objective 1) and over 20 different transduction enhancer compounds (Objective 2).

These have been refined to evaluate only the most promising candidates for in vivo (Objective 1: 3 HSC stem cell subsets; Objective 2: 3 Transduction enhancers).

Competitive transplant assays and the generation of mixed bone marrow chimeric mice have been included into the experimental plan where possible, as a method to provide a more robust answer of experimental vs control comparability, whilst providing an 'internal experimental control'. This further reduces the need for an independent control group.

The NC3R's Experimental Design Assistant has also been used to review and inform the experimental plan. Establishing pilot experiments to ensure experimental, technical success, minimise failures

What other measures apart from good experimental design will you use to minimise numbers?

Initial pilot studies will be performed to validate the experimental protocols, to ensure no unanticipated effects on animal well-being are observed and that all anticipated readouts can be achieved. This will avoid unnecessary experiment repetitions.

Secondary bone marrow transplants of human stem cell engrafted NSG mice are frequently performed as a gold-standard test to confirm the long-term repopulating potential of primary engrafted cells. To minimise the numbers of animals required, we will harvest bone marrow and perform secondary transplants from mice used in other experimental series (example, use bone marrow harvested from mice transplanted with the top cell dose in a limiting dilution experiment used to determine HSC Scid Repopulating Frequency).

Where possible, serial in-vivo imaging of individual mice will be used in order to reduce the number of animals killed for histology at various time-points.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mouse models of human and mouse cell transplants will be performed to address the aims of this Project. Cells will typically be transferred by intravenous injection, into mice which



are conditioned to receive cell transplant. Non-invasive imaging will be used to track cell behavior in vivo.

These procedures are minimally invasive and designed to reduce the likelihood of any adverse effects associated with toxicity or immune susceptibility as a result of conditioning treatments, or engraftment of mouse or human cells in vivo.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Adult mice are required to allow monitoring of long-term bone marrow engraftment and stem cell function, in an in vivo physiological setting that permits human and murine mouse cell growth and differentiation. Other less sentient models are not available to perform these observations.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The procedures planned are minimally invasive and refined to prevent and minimise discomfort or harm, pain and suffering:

Use of split dose irradiation techniques to minimise potential adverse effects

Daily monitoring of mice following cell transfer to ensure mice engraft and fully recover from the procedure

Use of non-invasive in vivo imaging techniques

Use of early humane study end-points using scoring charts to monitor animal health and wellbeing

inhalation anaesthesia will be preferentially used where possible for imaging, to aid more rapid animal recovery

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We have designed the protocols that will be used within this project by consulting Best Practice Guidelines published by LASA, FRAME and NC3Rs resources. These will be annually reviewed for updates throughout the project duration.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I will stay informed about potential refinements I can make and implement into the planned work in this Project, I will refer to published studies, scientific congress, and discuss with an NC3R representative. I will also conduct a mid-project review and report back to our AWERB for advice.

LASA, FRAME and NC3Rs resources will also be reviewed to update procedures and methods being used to address the Project aims throughout the study duration.



Explain the choice of species and the related life stages

Engraftment and the ability to reestablish blood cell production for the lifespan of a recipient is currently the gold standard definition of a stem cell (Rossi, CellStemCell. 2012). Critical information such as the survival of transferred immune cells in a host system, the migration of cells into disease affected tissues and impact on recipient survival can only be studied in an animal model. It is required by the UK and EU regulatory authorities (MHRA and EMEA) that advanced therapy investigational medicinal products (ATIMPs) are pre-clinically tested in animal models prior to phase I/II clinical trials in humans. The data generated in this project will inform and refine future pre-clinical studies that support regulatory requirements for ATMPs. The use of juvenile-adult mouse strains, with genetic backgrounds that permit human and mouse cell engraftment, and allow study of a single gene knock out and its restoration, are therefore the only models available to research and yield robust and highly relevant data sought in the Aims and Objectives of this Project. The choice of these models and animals will therefore provide important data to further the development of stem-cell based gene therapies for clinical use.





152. Studying central nervous system repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Mice	neonate, juvenile, adult, aged, embryo, pregnant
Rats	neonate, juvenile, adult, aged, 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 is the aim of this project?

To investigate the cellular and molecular mechanisms that control central nervous system repair in order to identify new therapeutic strategies for treatment of neurological 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?

There are currently no approved treatments aimed at repairing the central nervous system in neurological disorders, due to the lack of understanding of the mechanisms that control repair success and failure.

What outputs do you think you will see at the end of this project?



We aim to identify new targets for drugs to repair the brain following injury, more specifically to regenerate the insulation surrounding nerve fibres which is required for nerve health and function. We are achieving this by studying the contribution of immune cells in the brain which we have shown support this regeneration. At the end of this project, we will have a better understanding of the way in which the immune cells need to be stimulated in order for the brain to be repaired, which subtypes of immune cells need to be targeted, how these are involved in various neurological disorders, and what proteins can be used as a starting point for the design of new drugs to stimulate brain repair.

Our NC3Rs funded studentship also aims to develop a platform that could be widely adopted for screening in tissue explants, here focusing on screening for regenerative drugs in the context of the central nervous system using brain explants, with implications for use of these protocols in screening in either different neurological models in explants or in other organ explants. We predict that the development of this platform will reduce the number of animals needed in comparison to treatment of live animals, by 28 fold.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

We anticipate that both scientists and the general public will benefit from these outputs. First, other researchers in the field will benefit from the new knowledge we generate in understanding how brain repair takes place, and how it fails. This will aid in advancing the field towards new therapies. Scientists working directly on the project will also benefit in supporting their professional advancement (e.g. in doctoral studies). Second, patient groups for neurological disorders in which brain repair fails may eventually benefit from this research, as our goal is to identify ways in which drugs could stimulate repair. This is expected to be a long-term goal.

How will you maximise the outputs of your work?

We will maximize the outputs of this work by: 1) forming international collaborations with researchers with expertise in advanced techniques (e.g. drug delivery) and with useful models to advance our research, 2) disseminating the data via presentations at conferences and publication of results, 3) public engagement via interaction with charities REDACTED and social media 4) providing information on our research on our lab's REDACTED website. All large-scale data such as gene transcriptomes are made publicly available on online databases for all researchers to access for further study.

Species and numbers of animals expected to be used

- Mice: 9500
- Rats: 4500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Some animals will be used for breeding to generate animals that are used for subsequent experiments. Some animals are used for isolation of cells or central nervous system tissue for investigation. Some animals undergo experimentation to induce a small brain injury by injection of a substance, which normally does not lead to any signs of damage, pain, or distress, which we can then use to investigate the brain repair process. In some cases we used aged animals which show poor brain repair, and these also undergo the small focal brain injury approach. Some animals are genetically modified to allow us to visualize cells, modify gene expression, isolate RNA, or induce brain pathology. Most of our experiments are short term e.g. 1.5-3 weeks long. For experiments on aged animals, the oldest animal used is 15 months old.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We have extensive experience with inducing the small focal brain injury in animals and very rarely observe any adverse effects on the animals. Occasionally we will observe an undoing of sutures over the wound, which we can easily correct (once only). Occasionally we may observe temporary signs of pain or distress the day after surgery, at which point we consult the veterinarian and anti-inflammatory drugs may be administered or the animal culled if advised.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

We expect the proportion of animals to experience the following level of harm in each protocol:

Protocol 1) For both wildtype mice and rats, 75% will experience moderate severity due to exsanguination, perfusion-fixation, or use of transgenics with adverse clinical presentation.

Protocol 2) For both genetically altered mice and rats, 25% are expected to show moderate severity in clinical presentation due to elimination or overexpression of genes.

Protocol 3) For in vivo stereotaxis of mice and rats, 100% are expected to show moderate severity following surgery as all animals must undergo full anaesthesia and injection.

Protocol 4) For in vivo stereotaxis of juvenile mice, 75% are expected to show moderate severity as this proportion will undergo stereotaxis and/or hypoxia.

Protocol 5) For widespread demyelination with cuprizone in mice and rats, 50% are expected to show moderate severity from demyelination as some mice are on normal diet.

Protocol 6) All transgenic mice used for neurodegenerative models are expected to have moderate severity due to neural pathology.



What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Using animals allows us to i) determine the mechanisms involved in brain injury and repair, ii) do precise temporal analyses of events to understand the process of injury and repair, iii) manipulate the system to understand the contribution of a specific cell or protein in injury or repair, and iv) test the efficacy of drugs in supporting brain injury. There is remarkable conservation of events, cells, and proteins in rodents and in humans, making them suitable models to progress our knowledge in the treatment of neurological disease.

What was your strategy for searching for non-animal alternatives?

We considered the use of organoids ('balls' of central nervous system tissue) and skin cell-derived brain cells/ human embryonic stem cells.

Why were they not suitable?

Although some organoids have recently been shown to recreate some parts of the central nervous system, these do not have myelin or white matter and are thus not appropriate for our studies. In addition, it is not possible to only use fixed human post-mortem tissue to address our hypotheses as it is impossible to manipulate this tissue by performing gain or loss of function experiments or to determine the role of cells or molecules in central nervous system myelin repair. Additionally, although studies in other fields are now focusing on deriving stem cells from patient skin cells (iPS cells) or human embryonic stem cells then differentiating them to cells of interest, this is not appropriate for our studies because myelin repair involves the interaction of multiple cell types (thus increasing the difficulty of reliably generating all of these cells and ensuring their physiological interaction) and it is not currently understood whether these derived cells recapitulate all of the functional and gene expression profiles of in vivo-derived counterparts. Moreover, currently human brain cells cannot be produced in large numbers, limiting experiments that can be performed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 proposed was arrived at by our previous power calculations determining that to achieve a power of >80% at a significance of <0.05, we require 6 animals per group for in vivo experiments, and 12 animals per group (1 litter) to achieve a



significant number of cells or explants for tissue culture. We anticipate using more mice than rats because we use primarily mice for our brain explant and in vivo work, and rats only for a subset of experiments (less than 1 experiment per month per lab member).

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We will also use in vitro and ex vivo cultures for initial experiments before performing in vivo experiments, and use a shared control group between treatment groups. We also use statistical analysis of data to have statistical precision in our analyses. For live imaging, we reduce the number of animals required by a large amount due to the capacity to track the same animals over time within an experiment.

What other measures apart from good experimental design will you use to minimise numbers?

When possible, we will obtain tissue from other groups for models of interest, to avoid using additional animals. This allows us to test initial hypotheses and design pilot experiments. Pilot experiments use a small number of animals, e.g. 3, from which we use the data to perform power calculations to determine the maximum number of animals needed to have confidence in the result. Our breeding strategies also result in either using both sexes for experiments, or using some of the mice for breeding rather than 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice will be used for in vitro, ex vivo and in vivo models due to the availability of transgenic mice that will allow us to better answer the biological question at hand, the already established protocols of brain slice generation and induction of focal brain lesions in mice, and the ability to compare our data to the literature given that the majority of experiments in the field of myelin repair are conducted with mice. We employ models of focal myelin injury to facilitate analysis of repair (known location and time of induction) and due to focal lesions being observed in both multiple sclerosis and cerebral palsy. In addition, inducing focal lesions is the most refined method to use as it induces small lesions with mild clinical effects in comparison to models with more widespread damage (such as experimental autoimmune encephalomyelitis). We will use reporter mice expressing fluorescent proteins under the control of different cell-specific promoters and these are not known to have any detrimental phenotype. Other transgenic mice expressing mutations or variants of different myelin genes, or signalling molecules thought to be involved in myelin repair, are excellent ways of studying the biological processes involved in myelin repair by altering expression of the molecules involved. These may have phenotypes, but as we are interested in improving myelin repair after injury, we hope that they will regenerate quicker and thus suffering is reduced. In some instances, the use of conditional knockouts in which immune



cells (e.g. macrophages) have impaired function may be used, these may be immunocompromised therefore we will use individually ventilated isolator cages with germ-free food and water to prevent infection and suffering.

Rats will be used for the majority of the generation of in vitro cultures of central nervous system or bone marrow derived cells because we can generate higher yields of cells from these vs the mouse, and thus will reduce the number of animals required for each experiment. As with mouse studies, the protocols to isolate and maintain these cultures are already established, further reducing the need for optimization and the number of animals required.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Previous experiments have demonstrated the similarity between rodents and humans in terms of cells, cell behaviour, biological processes, and gene/protein expression.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We will monitor animals regularly, provide post-operative care including pain management, heat management, and providing mash on the floor of the cage, and will consult the veterinarian if any signs of pain or distress are observed. Animals will be weighed regularly and those showing >20% weight loss will be culled.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will plan studies to ensure we can publish following ARRIVE guidelines.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

I will check the NC3Rs website for any advancements in techniques available, will participate in the annual REDACTED NC3Rs day, and will send a member of my lab to the NC3Rs annual meeting in the UK.

Explain the choice of species and the related life stages

We study brain repair in various models of neurological disorders across the lifespan, including developmental brain injury (e.g. leading to cerebral palsy), brain damage in young adults (e.g. multiple sclerosis) and in old age (e.g. Alzheimer's disease and neurodegenerative disease). Thus we use neonatal and juvenile animals, adult animals, and aged animals. In addition, some cell types that we study are available in larger numbers in younger animals, and we can therefore use less animals in our research if we isolate our cells from young animals. Furthermore, we can grow slices of brain in a dish which allows us to investigate brain repair more easily than in a live animal, and these slices survive better when isolated from neonatal mice. Altogether this also reduces the amount of live experimentation done in animals.





153. Studying drivers of tumour heterogeneity and drug resistance

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

No answer provided

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 is the aim of this project?

To generate and demonstrate the utility of a novel transgenic mouse line, in which tumours will more closely resemble those that occur in humans than is currently achievable using existing mouse models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A significant challenge in cancer treatment is the enormous genetic variation seen within a single tumour, meaning that even if a therapy successfully kills the majority of cells in the tumour, some will be inherently resistant to that therapy. We are studying a group of genes ('REDACTED') whose normal function is to defend our cells against viral infection but



which can go rogue, causing errors (mutations) in our DNA and driving cancer development and/or progression during treatment by generating this 'genetic heterogeneity' within tumours.

REDACTED appear to play a key role in the development of numerous cancer types, including breast and lung cancer, which between them account for approximately 100,000 cases of cancer per year in the UK. The role of REDACTED in cancer has only been revealed in the last 5 years, due to our ability to sequence DNA from tumours and to identify the mutations that have caused these tumours to develop.

While much has been learned about other important cancer genes from studying them in mice (leading to many new cancer therapies), unfortunately mice have only one REDACTED gene, while humans have seven – therefore we cannot learn very much about how these genes work, or test anti-cancer drugs that are being designed to target specific human REDACTED genes by studying normal mice. To address this problem, we have generated a 'humanized' mouse line, in which we have replaced the one mouse REDACTED gene with part of a human chromosome containing all seven human REDACTED genes.

We predict that this humanized REDACTED mouse will bring at least three key benefits:

The ability to study the biology of the REDACTED genes within the context of a whole organism; increasing our understanding of basic biology, immune response to viral infections and cancer development.

The mice will serve as a key model in which REDACTED inhibitors currently under development for cancer therapy can be tested prior to entering clinical trials in humans.

Due to the mutagenic activity of the human REDACTED genes, these mice should serve as a background in which tumours that are more 'human-like' can be modelled, therefore serving as a more refined research tool for the broad range of cancer researchers and those engaged in cancer drug development who rely on the use of mouse tumour models.

What outputs do you think you will see at the end of this project?

We will establish whether this new mouse line is a useful and improved model for studying human cancer development.

We will determine whether these mice will be useful for the preclinical testing of anti-cancer drugs targeting our genes of interest ('REDACTEDs'). If so, our collaborators in drug discovery will use these mice to help bring their drugs to the point at which they can be trialled in human cancer patients.

We will publish a paper describing this new mouse line, detailing how it can be used for studies into viral infection, inflammation and cancer.

We will present our findings at national and international cancer conferences.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?



In the short / medium term (during the project) we will know if this mouse line is a useful tool for studying REDACTED biology during viral infection and cancer development. Once we know this, we will publish our findings and make the mice available to the wider research community.

In the longer term, we anticipate that this mouse line will become a very important tool for studying tumour development, used by many researchers and also by those in academia and industry, that are developing and testing cancer therapeutics.

How will you maximise the outputs of your work?

We are already collaborating with researchers engaged in drug discovery (REDACTED). We will publish and present our detailed characterisation of this new mouse model and make the mouse line itself widely available to the research community, through deposition with repositories (e.g. European Mutant Mouse Archive and/or Ximbio).

Species and numbers of animals expected to be used

- Mice: 1400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Adult mice may be injected once with a drug and monitored for up to 48 hours before being euthanised. Only drugs that have previously been administered to mice and for which safe doses have been established will be used in this study.

Other mice will be kept alive until old age (up to a maximum of 2 years) and will be closely monitored to check for signs of cancer development. Any mice displaying signs of discomfort/distress due to symptoms associated with ageing or cancer development will be euthanised immediately.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Mice injected with drug are expected to experience mild and short-lived pain at the time of injection but we don't anticipate any longer-term discomfort.

Mice that are aged to monitor for tumour development may experience symptoms associated with ageing including dry/flaky skin, possible hearing loss, problems with vision, digestive complications, problems controlling body temperature, excessive weight gain or loss, increased risk of tumours, abscesses, incontinence or problems urinating. Mice will be closely monitored for all such symptoms and will be either treated or euthanised. Mice will not suffer with any such symptoms for more than a few days at most.



Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Most (80%) of mice will be used either for breeding or for injection and these will experience no more than mild discomfort.

The approx 20% of mice that are used for ageing / tumour development may experience discomfort of moderate severity.

What will happen to the animals at the end of the study?

- 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 understand how tumours develop and how our genes of interest ('A3s') contribute to this process in the context of a living organism. Factors such as inflammation, disturbed sleep cycles and obesity can all increase our risk of cancer development and it is impossible to model these in cells growing in culture.

We also need to develop an animal model in which new drugs targeting the A3 genes can be tested before being trialled in humans.

What was your strategy for searching for non-animal alternatives?

We have conducted extensive analysis of the A3 genes in human clinical samples using DNA sequencing and gene expression data from The Cancer Genome Atlas project and International Cancer Genome Consortium. We have also made extensive use of human cell culture models, which form the main focus of our current research and we will continue to use human cell cultures wherever possible. We have invested a lot of effort in generating A3 knockout and A3-tagged human lines. Wherever possible we will use cells derived from our mice rather than the mice themselves for studying how the A3 genes are regulated in our new mouse line, thus sparing mice any unnecessary discomfort.

Why were they not suitable?

Human cell cultures will continue to be our primary research tool and will inform our animal experiments. However, the key questions concerning the role of A3 genes in cancer development and progression are only addressable in an animal model. Furthermore, the development of A3 inhibitors as anti-cancer agents depends upon establishment of appropriate preclinical models for testing prior to trialling in human cancer 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?

Most of our mice will be used for breeding. For the experimental mice, we have conducted power calculations to determine the minimum number needed to provide meaningful data.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We've followed guidelines detailed in the 'The Designing of Animal Experiments: Reducing the use of animals in research through better experimental design' (Festing, Overend, Borja and Berdoy; 2nd Edition).

What other measures apart from good experimental design will you use to minimise numbers?

We will use a pilot study to examine whether our ageing mice are tumour-prone. We will collect multiple tissues from euthanised mice to learn as much information as possible per mouse. We will also derive and culture cells from mice and will use those for analysis rather than performing procedures on live mice wherever possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will exclusively use mice for this project. In experiments in which mice are administered with drugs, each mouse will only experience this once and will be kept alive for a maximum of 48 hours post-administration, so will avoid any chronic/longer term effects.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We need to study tumour development and the genes in which we are interested act in humans over many years to generate the mutations in DNA that eventually cause cancer in adults. These genes are not relevant to paediatric cancers, so we need to study tumour development in the adult mouse. For experiments in which we will measure changes in gene expression following administration of drug, we need to evaluate these changes over a period of up to 48 hours, so it is not possible to perform this under terminal anaesthesia.



While a number of cancer-relevant studies can be conducted in non-protected animals, such as nematodes or flies, modelling the complex interplay between REDACTED gene expression and inflammation and/or viral infection and cancer development requires a model as close to humans as possible, hence our use of mice. Furthermore, we anticipate using these mice as a preclinical model for REDACTED-targeted drug development; again it is desirable/essential to conduct such studies in an organism that best approximates the action of a potential drug in patients, while avoiding the use of (for example) non-human primates.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

All work will be conducted by trained personnel, who will be given further training in specific techniques where necessary. This licence contains only protocols categorised as mild or moderate and anaesthesia and/or analgesia are used wherever appropriate. Animals will be closely monitored during all procedures and where possible we will consider refining existing techniques or incorporating new methods to minimise any suffering to the animals.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Ullman-Cullere et al 1999 'A Rapid and Accurate Method for Assessing Health Status in Mice' Lab Animal Science; 49(3):319-323.

Wilkinson et al 2019 'Progressing the care, husbandry and management of ageing mice used in scientific studies' Laboratory Animals 0(0):1-14.

Turner et al 2011 'Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider' J Am Assoc for Laboratory Animal Sci 50;600-613.

Workman et al 2010 'Guidance for the welfare and use of animals in cancer research' Br J Cancer 102;1555-157.

Diehl et al, 2001 'A good practice guide to the administration of substances and removal of blood, including routes and volumes' J. Appl. Toxicol. 21, 15–23.

'The Design of Animal Experiments: Reducing the use of animals in research through better experimental design' (Festing et al 2nd Ed).

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Regular attendance at local AWERB meetings, liaising with our vet, reading the latest relevant scientific literature, attending conferences and consulting policies on animal research from our funder (Cancer Research UK) and other funding bodies.

Explain the choice of species and the related life stages

We are using mice as they are a long-established and very useful model for studying cancer development in humans. The mouse is also a tractable system for conducting the complex genetic manipulation involved in introducing the human REDACTED locus.





154. Support procedures for Neural Repair

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo
Rats	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 is the aim of this project?

To breed normal and genetically altered animals to provide animals and tissues in a timely fashion for research into neural repair and protection, and perform initial testing of therapeutic compounds for compatibility.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 licence supports the work of a group working to develop methods to repair and protect against damage to the nervous system. The licence provides normal and genetically altered animals and tissues to support this work. The licence also allows initial testing of new therapeutic compounds to find out if they are compatible with mammals.

What outputs do you think you will see at the end of this project?

This is a licence to support the research of the group by providing animals and tissues in a timely fashion to enable research aimed at developing new treatments for damage to the



nervous system. It also allows for the initial testing of new treatment compounds to assess their compatibility.

Breeding according to the principles of demand-matched supply will minimize wastage and reduce the need to obtain lines from commercial suppliers thus reducing contingent transport stress.

New potentially therapeutic substances will be tested for compatibility with animals.

The outputs will be achieved through other licences held within the group. These will be through scientific publications relevant to the repair of the protection and repair of the nervous system.

Data will be published from the *in vivo* biocompatibility tests in the licence.

Tissue from animals kept in this licence is used for *in vitro* tests whose results will be published.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The main output of the licence will be the enabling of research within the group through the timely provision of animals and tissues for research and through initial compatibility testing of compounds.

Published data from biocompatibility testing and on any new tests developed will be used by academic and pharmaceutical company groups.

The overall aims of the group are to develop new treatments for spinal cord injury, Alzheimer's disease and glaucoma.

For spinal cord injury the aim is to develop new methods to promote regeneration of axons in order to restore function.

For Alzheimer's disease the aim is to develop treatments to reactivate plasticity in the adult CNS. This enables the brain to make new circuits to bypass the damaged neurons. This restores cognitive ability and delays the onset of dementia, but does not stop the slow progression of the neuronal damage caused by the disease.

In glaucoma the aim is to develop treatments that protect retinal ganglion cells from the effects of the disease and to enable damaged axons to regenerate in the optic nerve.

How will you maximise the outputs of your work?

The output of this licence will be published in papers from the group, enabling future collaborations and new models for investigation. Any new or improved methods of animal husbandry, production of transgenic animals and unsuccessful approaches will be published in papers from the group. The research group collaborates with several other research groups in various countries. Transgenic animals can be transferred from other groups or to other groups to enable research. Members of the group work closely with other groups worldwide and attend meetings with other groups working in the same area. Successful and unsuccessful methods are regularly discussed and presented.



Species and numbers of animals expected to be used

- Mice: 4740
- Rats: 1850

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Procedures are included that enable preservation of transgenic embryos and establishing transgenic lines from preserved embryos. These procedures involve minor surgery for vasectomy and implantation of embryos.

Most of the animals kept on the licence are transgenic or normal animals that are bred so as to provide the number of animals that are needed to provide cells for tissue culture, or to provide transgenic animals that will be transferred to other licences for experiments to discover new treatments for the repair and protection of the nervous system.

Some animals are used in pilot studies to test the compatibility of new potentially therapeutic compounds

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Procedures for preservation and restoration of transgenic lines. In these protocols animals receive minor surgery for vasectomy and embryo implantation. In rare cases there may be wound infection or the wound may need to be resutured once.

Maintenance and breeding of transgenic animals. The majority of transgenic animals kept under this licence have no deficits and behave like normal animals. Two lines that are models of Alzheimer's disease and Motor Neuron Disease are kept. These animals do not show any deficit until they are over 4 months old, during which time breeding is accomplished. In rare cases pregnant animals may be kept while showing adverse signs until they have delivered their litter, but in they are always killed before they become severely disabled.

Testing of compounds for compatibility. All compounds to be tested have been shown to be non-toxic and efficacious in tissue culture experiments, and past experience tells us that these compounds will be well tolerated by test animals with no 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 species)?



The animals used for generation of transgenic lines receive an anaesthetic and therefore come into the moderate category; they are expected to make a rapid recovery.

Over 90% of the transgenic animals show no deficit and are therefore in the mild category.

Animal models of Alzheimer's disease and Motor Neuron Disease can show early signs of poor mobility and weight loss. They are almost always used at a time at which the deficit is very small. Occasionally pregnant animals may be allowed to survive to a greater degree of disability within the moderate severity in order to deliver their litter.

Protocol 1. Mild all animals

Protocol 2. Moderate all animals Protocol 3. Moderate all animals

Protocol 4. Moderate approx. 30%, the rest Mild

Protocol 5. Non-recovery

Protocol 6. Mild all animals Protocol 7. Mild all animals Protocol 8. Mild all animals

Protocol 9. Mild all animals

What will happen to the animals at the end of the study?

- 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?

In order to discover new treatments to preserve and repair the nervous system, mechanisms and treatments are tested in tissue culture. Appropriate animal tissue is needed for this work. When promising treatments are identified they need to be tested in animals to show whether they can repair or protect a real nervous system with all its complexities. Tissue culture models are not yet adequate to determine whether treatments may work.

What was your strategy for searching for non-animal alternatives?

Most of the work in the group is performed in tissue culture. Only final verification is done in animal models.

Why were they not suitable?

The mammalian nervous system is extraordinarily complex, and subject to effects from the immune system and other body mechanisms. Only by testing treatments in whole animals can treatments that might be taken forward for use in human and veterinary patients be identified.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to



design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The purpose of the licence is efficient and timely supply of animals for tissue culture and work on other licences. The required number for animals for planned experiments are calculated on those licences. Numbers of transgenic lines must be sufficient to maintain a breeding population.

Numbers of animals used for establishing and preserving transgenic lines are calculated based on the likelihood of new lines being introduced, and the need to transfer or preserve lines if the lines are sent to other animal facilities.

Numbers of animals for provision of tissues is based on the current level of use of animals within the group.

Numbers of animals for testing of compatibility is based on initial testing of five new compounds during the time of the licence.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Breeding is carefully monitored so that sufficient but not excessive numbers of animals are bred for the planned experiments.

For pilot compatibility studies only 4 animals per group are planned, and animals will be dosed one at a time, so that if the first animal shows a reaction the experiment can be stopped or replanned.

What other measures apart from good experimental design will you use to minimise numbers?

In this licence efficient and timely breeding is performed. The reduction methods for the experimental work are described in the relevant licences.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Most of the animals used in this licence have no adverse phenotypes. Two transgenic lines are used to model Alzheimer's disease and Motor Neuron Disease.



In the Alzheimer animals, the genetic alteration is chosen so that animals show a deficit in memory two or more months before they become disabled. Experiments can therefore be performed on animals that appear normal.

In the Motor Neuron Disease model, animals are killed to provide cells for tissue culture before any motor deficit occurs.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Fish, insects do not give very good models of neurodegenerative disease or recovery of function compared to mammals. The tissue culture methods for which this licence provides tissue only work well with mammalian cells.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The procedures have already been highly refined based on work over many years, and very few animals show signs of harm. Tests are being further refined to allow testing of treatments at early ages, before any harmful deficit is apparent.

For compatibility testing of compounds, all compounds are screened for toxicity and efficacy in tissue culture studies. Testing begins with a low dose in a single animal and only escalates if no toxicity is seen.

When GA animals are transferred from elsewhere, where practicable germ plasm will be imported instead of live animals.

Where urine is to be collected, consideration will be given to using Lab Sand rather than collection of urine in a gridded cage.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Animal testing is constantly being upgraded in the host laboratory and elsewhere. New methods will be incorporated into our practice.

Excellent information is available on the various websites listed above which are constantly updated with new 3Rs information.

Full and updated information for licence-holders is provided under the project-holders website.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The establishment offers continuous training and advice through the training facility and the animal facilities.

Excellent information is available on <https://www.nc3rs.org.uk> which is constantly updated with new 3Rs information. <http://en.3rcenter.dk>, <http://www.frame.org.uk>, <http://3rs.ccac.ca>, <http://www.ahwla.org.uk>.



Excellent information on procedures is available on <https://animalcare.ubc.ca>

Full and updated information for licence-holders is provided under the project-holders website.

Explain the choice of species and the related life stages

The main purpose of the licence is to provide transgenic and normal animals for use in experimental procedures in the laboratory. These animals are either used to provide tissue for tissue culture experiments or are transferred to other licences held in the group for experimental work.

For tissue cultures late-stage embryos or newborn animals are needed to provide nerve tissue that can survive in tissue culture

For animal experiments in the group on repair and protection of the nervous system adult animals are used, matched to behavioural and anatomical models optimised for testing repair and protection treatments.

For testing compounds for compatibility adult animals are used, because the compounds will be tested for their ability to protect and/or repair the nervous system.





155. Targeted immune and molecular therapies for cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Mice	juvenile, adult, neonate, embryo, 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 is the aim of this project?

We aim to study the immune and molecular mechanisms associated with the development and spread of cancer and to discover and evaluate novel biological, immunological, immune cell and molecular diagnostic and therapeutic treatments, including novel antibodies and antibody-drug/inhibitor conjugates. We are particularly focusing on tissue cancers such as malignant melanoma, breast and ovarian carcinomas, especially subtypes of these diseases for which there are few effective therapies available. Our studies will help us understand how our immune system which protects us from infections, interacts with cancer cells.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.



Why is it important to undertake this work?

Despite some promising treatments available in the clinic, tissue malignancies represent a major group of diseases for which limited effective therapies exist. Although surgery, radiotherapy, chemotherapy and adjuvant therapy have been used, tissue cancers in their advanced stages are notoriously resistant to conventional drugs and therefore present a major therapeutic challenge. Attention has recently turned to the development of novel immune and targeted therapies, including monoclonal antibodies, designed to fight tissue cancers. Immune and immune cell therapies for cancer have been the focus of many studies because tissue tumours are known to elicit immune responses resulting in immune cell activation. Several molecular pathways associated with cancer cell growth and a small number of tumour antigens have been targeted using molecular or immune therapies. Therapeutic antibodies are established in medical treatment against autoimmune diseases, transplant rejection and cancer. In cancer therapy, the high specificity of an antibody for its cellular target is expected to specifically seek out malignant cells expressing this target. This can result in cancer cell death by several mechanisms including blocking vital cancer growth functions, by immunological mechanisms or by antibodies bringing toxic payloads directly to cancer cells. Although some therapeutic interventions are already approved for clinical use in oncology, their potential for the treatment of tissue cancers is far from being realised and the mechanisms of action against tumour cells of many of these agents are not fully understood. The aim of our research is to understand the underlying mechanisms of immune responses to malignancy and to employ this knowledge to design, study and evaluate novel immune and targeted treatments that can help control cancer progression and metastasis and can ultimately benefit patients who suffer from cancer.

What outputs do you think you will see at the end of this project?

Expected Benefits

Dissecting the mechanisms of cancer pathogenesis and the recruitment and alterations of immune cell signalling in cancer will further our understanding of how tumours grow and how different components of the immune system (e.g. T cells, Dendritic Cells, monocytes and macrophages, B lymphocytes and their expressed antibodies, basophils, mast cells, cytokines, chemokines, secreted mediators of immune responses) respond to tumour antigen and inflammatory stimuli. We aim to examine ways of modulating these responses with the view of “alerting” the immune system informing novel immune therapy design. We will strive to dissect the molecular mechanisms underlying tumour growth and metastatic potential. In vivo models described herein, used in combination with in vitro, ex vivo assays and tissue and tumour organoid models, will contribute to the design and translational potential of therapeutic and biomarker approaches.

Monoclonal antibodies targeting a limited group of tumour-associated antigens and checkpoint targets provide clear survival benefits in several cancers. There is an urgent need to build and improve upon the well-documented efficacy of Rituximab, Trastuzumab, or antibody-drug conjugates (ADCs) such as Trastuzumab-DM1 (T-DM1) to benefit patients who can only receive palliative care. We are working towards developing novel antibodies for aggressive and incurable malignant diseases. An antibody generated by our team is undergoing a first-in-man clinical trial. Studies in this project licence will inform research on this and other antibodies of this class and may be pivotal in expediting and informing their path from the lab bench to the patient bedside. Research models described here are crucial to downstream evaluations for our most promising novel candidates and



are required to help gain regulatory approval. If successful, our approaches are set to lead to improvements in the efficacy of a wide range of therapeutic strategies, including antibodies already in clinical use in conventional non- optimised forms.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

We dissect the immunological and molecular mechanisms underlying tumour growth and immune surveillance and test these findings in vitro, ex vivo and in selected in vivo assays to evaluate their physiological significance. Our translational research teams focus on the discovery and evaluation of cancer therapeutics such as antibody and biological modalities, immune cell treatments and vaccination strategies. We also dissect the mechanisms of action of these interventions using a variety of in vitro assays and in vivo models. These studies have directly resulted in the progress of some of these strategies into pre-clinical development and clinical trials in patients. We are keen to continue and improve on our discovery and efficacy studies for cancer therapeutics. We anticipate that the findings, complemented with refined in vivo studies, will guide the discovery of more effective interventions. If our strategies prove efficacious, our research will have a significant impact on cancer care and directly lead to more effective treatments.

How will you maximise the outputs of your work?

We will strive to disseminate relevant research results to the general public in an appropriate form. REDACTED is active in public engagement initiatives and has a track record of public engagement and outreach activities through and interviews addressed to lay audiences including patient support groups, and public engagement events. She is also a member of the South East London Consumer Research Panel for Cancer. Public engagement through these and similar formats will be pursued. Where appropriate, we will also promote new findings from this project to the wider community and lay public by communication and education such as by issuing press releases in conjunction with the REDACTED press office, which will ensure dissemination through the institutional website and in the local and national press. Our antibody discovery, cancer immunity and antibody immunotherapy research has already featured in newsletters and press releases.

To ensure that our research will benefit the academic community, any data produced from our research will be presented through oral or poster presentations at international and national conferences. Additionally, we maintain strong collaborative links with international groups working within the fields of antibody engineering, cancer immunology and immunotherapy, sharing ideas, resources and data, all instigated and supported through different consortium initiatives, dedicated meetings, conferences and original and position paper publication projects. Interaction forums include the Antibody Society, the American Association for Cancer Research (AACR), EuroMabNet, the Gordon Conferences and others. These forums will continue to provide important means for dissemination of scientific knowledge to enhance our understanding of cancer immunology and support innovation and translation of new treatments.

Species and numbers of animals expected to be used

- Mice: 5600
- Rats: 2700

Predicted harms



Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically, animals will receive a tumour challenge followed by treatments which may include human immune cells and therapeutic interventions. Tumour growth is monitored very closely, and animals are humanely killed at the end of the experiment or when the tumours reach a certain size. Monitoring and sampling to understand the effects of treatments is conducted during the study and at the end of the study. The most invasive procedures are those of skin tumour and normal skin transplantation and tumour transplantation in the mammary fat pad, conducted under anaesthesia. The protocols used involve least pain, suffering or distress or lasting harm for the animals.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We will establish and study rodent models of cancer and assess the effects of various treatments on helping or preventing tumours from growing. The protocols used involve least pain, suffering or distress or lasting harm for the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

None of the intended procedures reach beyond the moderate level of severity. Procedures reaching moderate severity arise from the induction of the disease model (cancer) and are necessary to evaluate the effects of potential therapies.

What will happen to the animals at the end of the study?

- 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 the pathways that lead to the development of cancer and by using this knowledge to develop novel immunological, molecular and biological approaches to cancer treatment, and to evaluate these as diagnostic and therapeutic tools in appropriate disease models. The in vivo studies are essential to facilitate functional and efficacy assessments for the treatment of patients with cancer. A major aspect of our research incorporates at an early stage and throughout the lifecycle of this programme, the use of in vitro screening, selection, characterisation, functional, mechanistic and efficacy assays,



which serves to significantly replace, reduce and refine all proposed in vivo experimental systems.

Overall, the majority of our investigations are conducted using patient samples and human volunteer- derived cells, ex vivo and in vitro, and supplemented by genomic/transcriptomic and immunohistochemical evaluations of human specimens. When absolutely necessary to conduct experiments in vivo, we have chosen rodents as the experimental animal of choice as they have the lowest neurophysiological sensitivity while still having an immune system of comparable complexity to humans.

What was your strategy for searching for non-animal alternatives?

Our successful work conducted at REDACTED London has led to the establishment and validation of numerous assays and model systems which replace and reduce in vivo studies and are designed to elucidate the mechanisms by which therapeutics including antibodies exert their anti-tumour effects against cancer cells. Examples of some of our validated in vitro models and assays are:

Cell culture-based studies using patient specimens

In vitro tumour equivalent organotypic/organoid culture models

Cancer cell viability and functional assays to evaluate the impact of potential therapies on cancer cell functions

Assays to evaluate the ability of antibodies to activate immune cells to kill cancer cells

Microscopy assays to study cell functions and cell-cell interactions

Why were they not suitable?

The starting point and the majority of our investigations on cancer pathogenesis, immune responses and therapeutics discovery and functions are conducted using patient samples, human-derived cells, ex vivo and in vitro culture and mechanistic assays. We utilise cell culture and cultured organ models as much as possible to develop and evaluate our therapeutic strategies. However, characterisation in live animals prior to clinical translation is essential and often a requirement by regulatory agencies to ensure that medicines are safe and efficacious. The hypotheses generated can only be further examined using in vivo models. Routinely, we precede in vivo studies by thorough in vitro culture system assessments. Data obtained from these will help select the most promising therapeutic candidates for in vivo experiments, thus minimising and refining these. Together, in vitro and in vivo studies will help to build a clearer picture of pathogenesis, spread and immune surveillance in cancer and help evaluate key molecular and biological modalities and cell-based interventions for further development and clinical testing. Some in vivo experiments may be conducted in parallel with cell analyses in order to maximise derived information.

We wish to conduct a reduced and refined set of animal studies for the following reasons:

Due to ethical limitations of investigations in humans we depend on the use of rodents to perform significant and scientifically valid research and pre-clinical testing of therapeutics.

There is no adequate in vitro model to assess the value of targeting key pathways in tissue cancers. The complexity of the mechanisms involved in carcinogenesis, (e.g. interactions of cancer cells with immune system components, molecular, biological, immune and



immune cell components, cells, soluble factors and interactions between several cell types in particular microenvironments) cannot be faithfully reproduced in vitro, necessitating in vivo experiments.

Due to the biological complexity of our studies, animals must be used when measuring the effects of treatments and the consequences on remodelling and repair processes which result from changes in all the cell types within the body. The latter are in turn modulated by circulating agents such as cytokines, as well as circulating cells. These processes cannot, therefore, be modelled only in cell culture or in human organoid and organotypic culture systems.

Since molecular, chemotherapeutic and biological therapeutics (e.g. antibodies) may be sequestered in different parts of the body, it is necessary to study their effects, tissue sequestering, biodistribution, presence and retention in tumours, in various organs and excretory pathways, to assess likely function, efficacy, toxicity and dosimetry in advance of translation to clinical testing in patients.

Although animals must be used in this project, this research will not only lead to the design of more effective biological treatments for tissue cancers, but in the future, some of our methods such as those for generating antibodies from human tissues has the potential to eliminate the need to use further animals to generate antibodies for the treatment of many diseases, including cancer.

For any targeted therapies to progress to clinical development, major insights into their pre-clinical efficacy for cancer therapy can only be derived from the use of relevant animal models of local and metastatic cancers.

Therefore, while we endeavour to utilise cell culture and cultured organ models as much as possible in evaluating therapeutics, their characterisation in live animals prior to translation to the clinic is essential and often a requirement by regulatory agencies responsible for ensuring that medicines work and are acceptably safe and efficacious. Therefore, in vivo studies are a necessary step in translational research pathways, to afford a prediction of therapeutic potential before clinical trials in patients for whom limited therapeutic options are available.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These estimations are based on our previous experience with experimental design and on assessments by power calculations on the numbers of animals necessary to maximise the outputs of our studies, and to attain the statistical data needed. These are conducted before starting the experiments and are based on our existing work.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?



To ensure we use the minimum number of animals, great care is taken towards experimental design and assessment of statistical data needed before experiments. Only where necessary, pilot studies can determine optimal doses and conditions for subsequent functional and efficacy assessments. This reduces the number of animals we use. We have extensive experience with generation of relevant data sets using the minimal number of animals. Experimental data sets are usually repeated 3 times assessed for normal distribution, mean and standard deviation generated. Statistical significance of differences in data points is assessed by obtaining a p value (< 0.05 for statistical significance) using tests for independent samples such as the unpaired student's T test or the Wilcoxon-Mann-Whitney U Test for non-parametric evaluations. Our work has been discussed/ designed with statisticians from the research design team at our institution and peer reviewed by funders. The statistical methods of data analysis have been published in numerous peer reviewed journals.

What other measures apart from good experimental design will you use to minimise numbers?

In our research, to develop and evaluate our therapeutic strategies we routinely utilise human samples, cell culture and cultured organ models as much as possible. This ensures that any subsequent in vivo study design is guided and optimised through these preceding data. However, the hypotheses generated with human samples and cell-based studies can only be further interrogated with experiments using in vivo models; these are deemed essential for evaluations prior to clinical translation and often a requirement by regulatory agencies to ensure that medicines are safe and efficacious.

To optimise the number of animals used, pilot studies in smaller sample groups (e.g. 3-4 animals per condition) will be implemented to determine optimal doses and conditions for subsequent functional and efficacy assessments to ascertain that data are reproducible and enable humane endpoints to be identified. The data derived from pilot studies will feed into group numbers used for definitive studies such as therapy experiments within appropriate experimental timeframes and statistically significant endpoints. These can optimise the number of animals we use overall and minimise the chance of side effects. To maximise the information from each study, samples and tissues extracted are most often used freshly (e.g. for flow cytometric analyses); less frequently, samples are also snap frozen or embedded in paraffin for downstream evaluations and these are routinely shared across users, thus maximising the information derived from each animal and experiment.

In preparation for in vivo studies, we aim to also maximise breeding efficiency by careful planning and by keeping the numbers of pairs to the minimum required through crosses and monitoring of all breeders. With these, we ensure that breeding setups are carefully maintained until the required animal cohorts are generated prior to 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?



Rodents have been chosen as experimental animals as they have the lowest neurophysiological sensitivity while still having an immune system of comparable complexity to the human and are therefore the most frequently used animals in studies of human pathology/immunopathology. For example, in the context of antibody (IgE/IgG) therapies, rats have immune systems with similar IgE antibody Fc receptor distribution/expression on immune effector cells that closely mirrors that of humans; they are therefore the most clinically-relevant animals in studies of antibody function, safety and efficacy.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Solid tumours such as melanoma, ovarian and breast cancers normally manifest in adults. Therefore, adult rodents have been chosen as experimental animals. Rodents have been selected as they have the lowest neurophysiological sensitivity while still having an immune system of comparable complexity to the human and are therefore the most frequently used animals in studies of human pathology/immunopathology.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The protocols used involve least pain, suffering or distress or lasting harm for the animals. None of the intended procedures reach beyond the moderate level of severity. Procedures reaching moderate severity arise from the induction of the disease model (cancer) and are thus inherent to the disease studied. The limits of tumour sizes and endpoints for tumour growth are in adherence with recommendations published by Workman et al., 2010. There are no major invasive procedures except those of skin tumour, normal skin and breast tumour transplantation. These procedures involve well-established surgical techniques. Anaesthesia and analgesia will be administered to minimise discomfort and the animals will be monitored closely during all procedures and assessed regularly for any signs of distress. In all the proposed in vivo models, if animals display signs of distress, advice will be sought from the Named Veterinary Surgeon and, if distress cannot be alleviated, the animals will be humanely euthanized.

When it is necessary to conduct in vivo studies, our protocols involve established techniques involving the minimum of suffering. Anaesthesia and analgesia are administered to minimise discomfort and the animals are monitored closely during all procedures and assessed regularly for any signs of distress. In our in vivo models, if animals display signs of distress, advice is sought from REDACTED staff, NACWOs or the NVS and, if distress cannot be alleviated, the animals are humanely euthanised. To ensure we use the minimum number of animals in our experiments, we place great care is placed on the experimental design and assessment of the statistical data needed before starting the experiments. We routinely conduct pilot studies in order to determine optimal doses and conditions for our subsequent assessments.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The limits of tumour sizes, limits of interventions and endpoints for tumour growth are all in adherence with recommendations published by Workman et al., 2010. The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines which were developed as



part of an NC3Rs (<https://www.nc3rs.org.uk/the-3rs>) initiative to improve the design, analysis and reporting of research using animals – maximising information published and minimising unnecessary studies.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The REDACTED NC3Rs Regional Programme Manager is a point of contact with regards to developments in the 3Rs. These may include Experimental Design Workshops run at REDACTED. The Experimental Design Assistant (EDA) is a free resource from NC3Rs helps to design robust experiments more likely to yield reliable and reproducible results. Additionally, researchers under this licence will be kept up to date through the NC3Rs website (<https://www.nc3rs.org.uk/the-3rs>).

Explain the choice of species and the related life stages

Rodents have been chosen as experimental animals as they have the lowest neurophysiological sensitivity while still having an immune system of comparable complexity to the human and are therefore the most frequently used animals in studies of human pathology/immunopathology.





156. Targeting Biotherapeutics to Arthritic Tissue

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

Our study’s goal is to impact on the burden of musculoskeletal illness. We aim to develop improved treatment modality for arthritic conditions. We developed antibodies that specifically bind arthritic damage cartilage. We intend to use these antibodies to facilitate very early diagnosis of cartilage damage in the joints as well as to develop treatment that is designed so that drugs are specifically accumulated in the diseased joints to increase efficacy and reduce side effects.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Musculoskeletal conditions are typically characterised by pain and limitations in mobility. The most common and disabling musculoskeletal conditions are osteoarthritis and rheumatoid arthritis.



Rheumatoid arthritis characterised by long-term inflammation in the joints that leads to cartilage and bone destruction and, eventually, pain and deformation. Approximately 68% of rheumatoid arthritis patients in the UK are physically inactive creating a vicious cycle of disease progression and increased pain, thus affecting both physical and mental health. The sooner one starts treatment for rheumatoid arthritis, the more effective it's likely to be. Even though innovations in treatment strategies and monitoring are helping the patients, the high cost of drugs and limited health care budgets are restricting the treatment. Nevertheless, apart from adverse side effect of infections many patients still have partial response to treatment and 40% patients do not response to treatment. Therefore there is a need to develop new treatments that will be more effective and with less side effects.

Osteoarthritis is a disease of high incidence and prevalence worldwide and is the most frequent cause of disability worldwide. 10% of the world's population aged 60 years, or older, have significant clinical problems such as pain and reduced function that is attributed to osteoarthritis. In fact, pain is making a third of people with OA give up work or reduce hours. The only current treatment is joint replacement that is able to relieve pain.

Our study aims to develop a therapeutic that will minimise the unwanted side effect of treatments, facilitate the development of combined treatment and provide very early diagnosis for a better outcome. We will used models on mono arthritis to validate the potential therapeutic translation of our development, thus we can validate targeting to diseased arthritic tissue and compared to healthy tissue in the animal. The outputs of our study will be able to identify new therapeutic modalities for both rheumatoid arthritis and osteoarthritis in one hand and on the other hand early diagnostic tools that are lacking.

What outputs do you think you will see at the end of this project?

Our study's goal is to develop targeted treatments that will ultimately impact on the burden of musculoskeletal illness. Our study aim to develop therapeutic that will target treatment specifically to the effected joint and minimize systemic exposure to drugs. We expect this approach to minimize the unwanted side effect of treatments, facilitate the development of combined treatment and provide very early diagnosis for better outcome. The outputs of our study will lead to publications, patents and possible licencing by big pharma. Our research will lead to identification of new tool for targeting therapeutic modalities for both rheumatoid arthritis and osteoarthritis in one hand and on the other hand early diagnostic tools that are lacking.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Targeting drugs specifically to arthritic joint results in acceleration of resolution of the inflammation that is triggering the disease. Patients with rheumatoid arthritis will benefit from new drugs that will increase efficacy and reduce side effects associated with existing treatments. While several new drugs provide good results in treatment of 60-70% of rheumatoid arthritis patients, still 30-40% of patients do not benefit from these drugs. There are also safety issues due to the high risk of developing infections as a side effect of these treatments. There is therefore an unmet need to develop new strategies to improve the efficacy and the safety of these agents. The ability to direct treatment only to the effected joints will address at least some of these unmet needs

Our research provide early detection of joint disease, by detecting early onset of disease and before damaged of the cartilage can be detected by the classical methods such as



MRI and x ray. Our study may possibly improve efficacy as early treatment before any cartilage damage, especially if treatment can be localised only in the effected joints by our developed antibody platform. This research is also relevant to animal health. For instance, horses and some breeds of dogs suffer from disabling cartilage loss and arthritis. Therefore early diagnosis and repair is also eagerly sought after, particularly for horses.

How will you maximise the outputs of your work?

The aim is to maximize output publications in scientific journals, patents, worldwide presentations (I am regularly invited to antibody meeting, and is in the scientific advisory board in the biggest antibody meetings), engagement with pharmaceuticals with the aim to enter a clinical trials for new diagnostic and treatment modalities for arthritic conditions.
REDACTED

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mice model of inflammatory arthritis:

To mimic arthritis inflammation will be induce in a single knee. The contralateral knee will remain healthy as control.

Most animals are expected to develop arthritis: joint erythema and swelling in the inflamed joint. Pain is anticipated due to injection and is anticipated to increase as disease progresses

At any point after induction of knee inflammation, mice will be imaged (MRI /X-ray..). This will be carried out to monitor the disease progression using minimally invasive imaging.

At any point after induction of knee inflammation, mice will be injected with therapeutic compound(s). Mice will be monitored for efficacy of treatments by measuring the degree of knee swelling and in some cases by imaging.

These compounds may interfere with the induction of arthritis, or to the end of arthritis. Appropriate vehicle controls could also be administered in cohorts of animals. If a compound has not been previously tested a safety pilot experiment will be run.

Compounds will include:

Pharmacological compounds that are made of protein drugs the so call biologics or biologics that are loaded on scaffolds or cells.



At chosen time before and after the treatment, blood samples may be collected from mice from the tail vein. Alternatively blood samples will be collected after animal were cult. Animals will not return to the cage until certain that the bleeding has stopped.

The arthritis is typically self-limiting and its duration is usually about 7 days but varies depending on the genetic background and variability from experiment to experiment.

Mice model of osteoarthritis:

Incision will be made in the joint ligament in one knee. The contralateral knee will be operated but without knee resection or joint damage. Operation will be performed under anaesthesia. All procedures will be carried out under anaesthesia and with analgesia for pain management.

The development of the disease will be followed by minimal invasive imaging animals by MRI/PET/CT/X-ray to quantify the extent of cartilage damage and localization of the antibody we developed. Imaging would be performed under anaesthesia with maximal frequency of twice a day, only at day one after injection/operation animal may undergo a maximum of 20 imaging times (GA- generally ~10min sessions max).

At any point of time in the procedure, the animals may have compounds administered. These compounds may interfere with the induction of arthritis, or to the end of arthritis. Appropriate vehicle controls could also be administered in cohorts of animals. If a compound has not been previously tested a safety pilot experiment will be run.

Compounds will include:

pharmacological compounds that are made of protein drugs the so call biologics or biologics that are loaded on scaffolds or cells.

At chosen time before and after the treatment, blood samples may be collected from mice from the tail vein. Alternatively blood samples will be collected after animal were cult. Animals will not return to the cage until certain that the bleeding has stopped.

Duration of OA model will be up to 8 weeks.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Most animals are expected to develop one knee swelling due to the induced inflammation or mild destabilization of the joint ligaments.. In both cases pain is anticipated but animals will be monitored to ensure no progression beyond moderate impairment.

The use of anaesthetics carries an associated risk due to inappropriate depth of anaesthesia (<1% incidence), however, it is expected that animals will recover uneventfully from anaesthesia.

Discomfort or infection due to injection: Animals will experience stress due to restraint and transient discomfort from needle insertion. Redness/swelling of injection sites will indicate inflammation/infection. The injection may generate a local allergic reaction, but such events will be carefully monitored and if the injection causes the animals distress (e.g. scratching lasting more than 4 hours) the animal will be humanely killed.



Some animals may developed ulcer at the site of injection

Injection into the joint or ligament cut may lead to painful joints, sore feet, lameness, disability and distress.

Animals are expected to recover uneventfully from anaesthesia and surgery. Animals are expected to resume normal mobility and started to eat and drink by the end of the working day of surgery. Frequent care and welfare monitoring will be in place to ensure appropriate pain management, and if necessary –implementation of humane endpoints.

No adverse effects are expected following the administration of drugs, many of which e.g. are already used in the clinic.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

For the inflammatory model, most animals are expected to develop inflamed joint associated with swelling of the injected joint. The severity is mild to moderate depending on the developed knee inflammation. The severity of ligament disruption models in a single joint can go from hardly noticeable to moderate. Cartilage degradation is expected, with major cartilage degradation apparent at 4 weeks post-surgery. We will terminate the experiment 8 weeks after operation in order to be able to monitor sequential pathology while maintaining maximal moderate severity.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to demonstrate an effect on the immune reaction that occurs in the human arthritic joint, this needs modelling. This requires integrative systems that comprise the fully functioning immune and inflammatory components within the joint space. In addition to this, the joint needs to be damaged by these processes in order for the antibodies to bind and target the therapeutic. This complex combination of events can only be demonstrated in autoimmune/inflammatory models of arthritis, the therapeutics being chosen to affect auto-immune and/or inflammatory cascades.

What was your strategy for searching for non-animal alternatives?

Before performing any animal studies we will perform studies using human cartilage from hip replacement spares to make sure that the antibody and antibody-drug conjugate bind and penetrate the arthritic cartilage. We might employ organ culture for further tests as well.



Why were they not suitable?

Human post operation cartilage explants experiments can demonstrate specific binding but are not able to demonstrate specific accumulation of drugs that are injected systemically as well as not suitable to check drug efficacy of disease progression. Arthritic diseases are complex and involve interactions between cells leading to the disease manifestations that cannot be assembled in the test tube. Only animal models can reflect some of the complex pathology in human disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Most of our experiments will be performed in mice models of arthritis. This will include models of rheumatoid arthritis and models of osteoarthritis. Based on our previous studies, we have estimated around 40 mice for each experiment: usually we use 6 animals per treatment group to observe significant difference between treated and non-treated control group. We usually reach to 30 animal per experiments: 6 mice treated with antibody fused to compound 1; 6 mice treated with antibody fused to treatment 2; 6 mice treated with non relevant antibody fragment fused to compound 1; 6 mice treated with antibody fragment fused to treatment 1 and 2; 6 non treated control group. Rat will be used only for the osteoarthritis model to confirm results obtain in the mice and thus the expected number is small.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

To spare animal numbers, we will primarily use models where we induce the disease in one joint and use the contralateral joint as a healthy joint control. Hence, to save further animals use, most of our experiments will be carried out using one model of rheumatoid arthritis-namely induce inflammation in one joint. For osteoarthritis we will also use mostly a single model of osteoarthritis. A second model for bot diseases will be used for small group of animals to confirm the translatable nature of the results at the end point.

We will develop an imaging approach where we use our developed antibodies to longitudinally follow disease progression and response to treatment. This will save a large number of animals, as the same animal will be used for several time points and will improve reproducibility and reduce numbers. This is in contrast to current studies where animals are killed at each time point for disease/response validation.

In addition, to avoid excessive use of animals we will perform power calculations to ensure that we use the minimum number of animals needed to obtain statistically significant results. This will avoid excessive use of animals, whilst retaining statistical power. We will use a significance level of 5% and a power of 80% group sizes which are typically 6 animals per group. We will ensure randomisation of animals to different treatment groups and analyse outcome blindly as recommended in the ARRIVE guidelines. Experiment



designing, calculations and analysis of data will be performed in consultation with statisticians.

What other measures apart from good experimental design will you use to minimise numbers?

We have already a wealth of pilot data from our current experiments that will assist us in optimising the animal usage. We also plan to use human explant tissue to minimize the use of animal tissue for histology experiments. In our institute we have other groups that are working on these models and we can share some of the tissues from mice for additional histology study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The arthritis model that will be used in this study is the least severe model and the majority of our experiment will be done in this model. Once we see potent treatment efficacy of one of our candidate we will test it in a second model in a small group of animals which is also moderate. In both models, inflammation is only in one knee of mice. Similarly for the osteoarthritis studies most of our experiments will be performed using a single model which is a mild model of osteoarthritis which will follow by a second model in only small number of rat to confirm efficacy in a second model of osteoarthritis.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Joint diseases are complex and involve a great number of interactions between cells leading to the disease manifestations that cannot be assembled in the test tube or terminally anesthetized mice. We therefore use live animals to look at how disease progress in the whole organism, and how they may be corrected to stop the progression of the disease. No similar joints to humans are present in less sentient species.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals will be monitored regularly for signs of adverse effects including unexpected inflammation/infection, body weight, along with a body condition score will be monitored to ensure food intake is maintained. Behaviour and overall condition indicative of health status (activity, responsiveness, condition of coat, posture) will be assessed. Injection sites will be monitored for signs of redness, swelling and infection.



Imaging of diseased-induced knee will also be another indicator for degree of cartilage erosion which will allow us better predictability of disease progression and better management of clinical signs and thus animal suffering.

To minimize distress in arthritic mice we will follow the measures that include the provision of soft sawdust litter to reduce pain on walking, the use of non-tangling nesting material or long nozzles on drinking bottles.

To minimise stress animals will be carefully restrained and procedures will be conducted by trained and competent individuals.

A soft diet will be provided if there are concerns over the animal's ability to feed.

Inflammation at injection sites will be treated as advised with appropriate local analgesic and/or anti-inflammatory agents.

To minimize the risk of infection, animals of the cleanest health status should be used and held within barrier protection at all times. Analgesia will be used as advised by the NVS.

Animals will be monitored daily. Animals displaying diarrhoea will be supported via administration of fluids and/or an enriched diet for the first 48 hours to give them the opportunity to recover. After these treatments animals will be assessed to confirm they are fit to undergo further procedures.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

To minimize distress in arthritic mice we will follow the measures outlined in the publication "Applying refinement to the use of mice and rats in rheumatoid arthritis research" by Hawkins et al 2015.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Yes, keep updated by reading papers and attend expert conferences

Explain the choice of species and the related life stages

Our project aims at understanding the causes underlying the development of long lasting (chronic) arthritic diseases. In vivo studies, using animal 'models' of arthritis, are currently part of the research and development process for new or improved therapies and treatments. The rodent models we are using are very relevant to our study to test new treatment modalities as well as to validate the potential of our newly developed antibody to detect the onset of the disease at a very early stage and to monitor it longitudinally. We are primarily, but not exclusively focusing in our research on Rheumatoid arthritis (RA- arthritis associated to inflammation of joints) and Osteoarthritis (OA-generally more associated with physical overuse or direct damage in the joint cartilage). We have been researching how each of these conditions occurs and finding new methods for treating them.

We plan to investigate and develop new drugs capable of switch off inflammation and cartilage damage thus to achieve long-term cure.





157. Targeting Relapse Initiating Mechanisms in Adult Acute Lymphoblastic Leukaemia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The overall goals of this project are to understand more about the pathogenesis of acute lymphoblastic leukaemia (ALL) in adults, in particular to deliver major insights into relapse associated mechanisms (self-renewal, dormancy and increased survival) and to use this knowledge to develop novel therapeutics for ALL.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Adult ALL is an aggressive haematological malignancy and treatment consists of intensive induction chemotherapy followed by cycles of highly toxic drugs given in intensive inpatient cycles for 1-2 years. Despite the robust approach to treatment, long-term survival is achieved in only approximately 50% of adults. After relapse, few (<7%) survive. An



improved understanding of relapse-initiating mechanisms together with novel treatment approaches are therefore urgently needed.

What outputs do you think you will see at the end of this project?

This project will produce new understanding of leukaemia initiating cell biology which will in turn lead to new treatment strategies that can be developed further in clinical trials. The outputs of the project will be published in the scientific literature and disseminated at scientific conferences.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

It is intended that the ultimate beneficiaries of the outputs from this project will be patients with ALL through clinical trial implementation however the timescales for such an achievement will be medium/long. However, my role as a clinical academic and active clinical trialist in the field of adult ALL would lend optimal positioning to facilitate full clinical translation. A rather more short/medium term output from the project will be to generate new knowledge and innovation in a previously unexplored area, with potential for cross-disciplinary feeds since concepts conceived under this project may have wider implications for the field of leukaemia biology or indeed solid cancer and normal haematopoiesis.

How will you maximise the outputs of your work?

We follow the principals of research data sharing. Our data will be disseminated through conferences/workshops and publications in open-access journals. After publication, data will be deposited in an open access digital repository. Published materials/reagents/mice will be available upon request to allow for collaboration and dissemination of new knowledge.

We operate within both clinical and scientific professional local, regional and national networks. For example, REDACTED, National Cancer clinical subgroups and other similar forums. This allows for further collaborative working, sharing and efficient dissemination of results.

Raw data of potential use to others will be shared in public repositories, such as NCBI Gene Expression Omnibus, and experiential methods will be shared in detail.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



Animals will be housed in protective conditions, usually in groups of 5, with sufficient space to ensure best husbandry practice. Once they are acclimatised to our animal unit they will be injected with tumour cells and then monitored for both their welfare and also the development of the model of leukaemia. In some cases mice will be given a low dose of radiotherapy or chemotherapy prior to injecting the tumour cells to prevent rejection.

Mice selected for testing of new drugs will be given pre-specified courses of treatment and during this time their welfare will continually be monitored as well as the effects of the treatment on the burden of leukaemia. If mice at any stage are seen to suffer they will be killed in a humane manner; to achieve this we have a strict, objective scoring system to assess mouse welfare with a prespecified cut-off to prompt such action. We also pioneer a more sensitive method of measuring tumour burden in the mice and this allows for early start and endpoints to our experiments. At the end of the experiment the mice are killed and this allows us to collect the material used to analyse the experiment.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Animals will suffer only minor impacts from procedures such as handling, ear marking and blood taking. The impacts from other procedures such as preparatory radiotherapy/chemotherapy tumour injections, treatment regimes and bone marrow sampling are more significant and may include pain, weight loss, dehydration and lethargy.

However, through experimental design, driven by our experience with these models, the mice usually tolerate the procedures very well. We also have a comprehensive system of work including with supportive care throughout the experimental procedures to minimise the harmful effects to animals so that they are very rarely, if ever, distressed. Of specific note is that we try to use tumour samples that do not require the mice to undergo preparatory radiotherapy or chemotherapy but where this is necessary we use very low doses which minimise adverse effects.

We have very strict protocols for responding to mice who appear to be suffering to allow for swift interventions to prevent unnecessary levels of suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Our animals are expected to have moderate severities during our experiences.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?



Murine models are a key part of this work for two reasons:

First, the work concerns identification of leukaemia initiating (stem) cells and with current technology it is not possible to properly culture them in glass - these type of cells quickly die and/or lose their potential when they are plated out in dishes.

Next, the self-renewal hallmark of leukaemia stem cells can only be confirmed through serial transplantation in murine models.

That is, the leukaemia stem cells can only be identified properly by their ability to regenerate leukaemia, and this does not happen when they are kept in glass as opposed to in animal models.

What was your strategy for searching for non-animal alternatives?

A combined cell culture system that mimics some of the effects of the bone marrow environment on leukaemia cells has been developed and is utilised for initial experiments in which initial theories are tested before using mouse models.

Why were they not suitable?

The models described above are only suitable for some of the work; they are unable to recapitulate the biology of leukaemia initiating cells because of limited proliferation and survival of primary ALL cells outside of a living body. To be able to study the biology of these cells in close enough detail to be able to find mechanisms by which they can be treated we must use mouse models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our animal number estimation comes from careful planning of our experiments based on our own experience as well as that from other researchers. We ensure we use the minimum amount of animals to generate meaningful data which can then inform the basis for translation of our results to benefit humans with ALL.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Our experience and that of others has allowed us to design experiments in which the number of mice used is minimised and in which the duration and severity of the experiments are reduced as much as possible. Where relevant in the project design we have performed power analysis to determine the minimum number of animals required to robustly determine our defined experimental endpoint. We also incorporate "future proofing" measures into our experiments, e.g. by preserving DNA from the animals, so that it can be processed again without having to use more animals.



What other measures apart from good experimental design will you use to minimise numbers?

In vitro studies where feasible allow for minimisation of animals required for in vivo experiments.

Using engraftment pilot studies we avoid exposing a full cohort of mice to a leukaemia graft which is destined to not work and therefore give no usable outputs.

The experience of animal handling and interventions held by my research team is invaluable for the purpose of reduction. We have an exemplary record on mice welfare meaning that no mice are wasted, evidenced by our record of 0 found dead over the last year.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

During this project we will use immunodeficient mouse models.

I have extensive experience working with these animals to investigate the biology of ALL. During this time I have refined the steps involved in this type of research continually.

A refinement which I am leading on is the use of intratibial bone marrow sampling which allows for greater sensitivity in tumour assessment and this therefore allows for advanced detection of scientific endpoints prior to any clinical deterioration of the mice, when compared to more traditional blood sampling which we find to be less sensitive and less robust. The technique is performed with a very short general anaesthetic which allows for the minimum of suffering and a very rapid return to normal post-procedure.

These mouse models are the accepted standard for this type of research and are used across ALL research groups worldwide. We share methods via the scientific literature to ensure we are working with equal or improved welfare standards to our colleagues elsewhere.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice and humans share many biological similarities which makes it possible to model human leukaemia in mouse models. It is possible to study certain "highly conserved" biological mechanisms in less sentient animals, such as fish, flies or worms, but this does not extend to studying human leukaemia initiating cell biology. This means that data we gather from mice are applicable to humans and can potentially be quickly translated into human medicine; something which would not be possible from models generated in less sentient animals.



What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We use the least invasive method of tumour seeding possible in the least immunodeficient strain to allow the most efficient engraftment. For primary human tumour cell engraftment, our previous work has shown that intra bone marrow injection into NSG mice produces the most efficient tumour engraftment although intravenous methods may have a specific role in certain experimental contexts and for certain ALL subsets alternative routes, such as subcutaneous, may be required. Mice are anaesthetised during the procedure which proceeds quickly (<5 minutes) with minimal effect on the mouse.

All procedures performed by members of the team are done with strict supervision and after competence assessment by our NACWO/NTCO.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We observe both the PREPARE and ARRIVE guidelines for planning and reporting our work.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Professional development activities, organised locally throughout our REDACTED and collaborating institutions, such as the recent *REDACTED*.

We work closely with our NACWO and NVS team and all members of the team will periodically go to events and training organised by NC3Rs. We are continually looking to refine our models and reduce the amount of mice we use and so would quickly incorporate any new processes that are relevant to our research.

Explain the choice of species and the related life stages

We will use adult immunodeficient mice in our experiments, since a mouse with an immune system would quickly reject transplant cancer cells. Immunodeficient mice that allow the tumours to develop are the only option available for successfully creating models of ALL that allow us to further understand how these tumours grow and relapse. Likewise it also enables us to test new therapies targeted at these mechanisms. Previous mouse types for generating human leukaemia models were prone to developing mouse lymphoma but we are avoiding this by using a newer breed.

Where possible we are performing experiments in glass dishes or using computational models to avoid using more mice than necessary but for a detailed understanding of ALL growth and relapse the mouse del is required





158. Targeting the molecular mechanisms of 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
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged
Rats	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 is the aim of this project?

Our work in this project licence aims to first understand the molecular mechanisms of neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and motor neuron disease, and then to develop and test new treatments in rodent models of disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Degenerative disorders of the brain, such as Alzheimer's and Parkinson's, resulting in deficits in cognition and movement, are becoming more common as the population gets



older, as age is the greatest risk factor. The national census in 2011 revealed that there are more people over 60 than under 16 in the UK for the first time. The UK Government has responded to this through the Prime Minister's Dementia Challenge and the UK Dementias Platform. Neurodegeneration is not only a disease of ageing western societies, but a world-wide problem: there predicted to be more people in China with dementia by 2040 than the rest of the developed world put together.

There are currently 850,000 people with dementia in the UK, with numbers set to rise to over 1 million by 2025 and soar to 2 million by 2051. Currently another 140,000 suffer from Parkinson's disease, a number which will also rise with our ageing population. Work such as ours, starting in cellular models and moving to rodent models, is essential to develop and test new treatments.

What outputs do you think you will see at the end of this project?

Outputs from our work will include a new understanding of the mechanisms of neurodegeneration, new publications in leading scientific journals, and ultimately the development of new neuroprotective therapies.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Those in the scientific research community working on better understanding the mechanisms of neurodegeneration will be the first to benefit as new tools, models and experimental approaches are published in the short-term. We have published rodent models for Parkinson's (alpha-synuclein transgenic mice and LRRK2 transgenic rats), Alzheimer's (MAPT transgenic mice) and motor neuron disease (TDP-43 transgenic mice). All the rodent models we have generated and characterised are made available for all to access, for example our alpha-synuclein transgenic mouse models are made available through the REDACTED and have been taken up by several laboratories in academia and the pharma industry worldwide.

Longer-term beneficiaries will be the patients, carers and families of those with neurodegeneration who will benefit from improved neuroprotective therapies. In neurodegenerative diseases specific neuronal populations die which we aim to preserve. We are working on distinct approaches to develop novel therapies to preserve either dopamine neurons (lost in Parkinson's), cortical neurons (lost in Alzheimer's), motor neurons (lost in motor neuron disease) or sensory neurons (lost in Friedreich's ataxia). We work in close partnership with a number of industrial partners from pharma to discover and develop new therapies. Such partnerships will be powerful in ultimately bringing new treatments to the clinic. Treatment of several diseases in the clinic is made more difficult because the clinical symptoms of each disease only become apparent when the underlying neuronal changes are well underway. We liaise closely with colleagues working in the clinic who are developing methods for detecting pre- symptomatic phases of disease in patients using genetic markers of susceptibility, biomarkers and brain imaging. Once those prodromal phase individuals can be identified they will be targeted as most likely to benefit from future neuroprotective therapies. Prodromal disease includes mild cognitive impairment (MCI) as a prodrome for Alzheimer's and REM sleep behaviour disorder (RBD) for Parkinson's.

How will you maximise the outputs of your work?



Several different approaches will be used to maximise output of this work. In the first instance, we will disseminate new work through presentations of posters and talks at national and international conferences and then publish our work in academic journals. We aim to publish all our work, including those generating negative results. We also present our work to lay audiences through travelling to local interest groups, holding events and Open Days or through online resources, such as podcasts. We establish national and international collaborations which may form the basis of future funding opportunities. Finally, we engage with industry which can be a means of further developing or funding translational work.

Species and numbers of animals expected to be used

- Mice: 34000
- Rats: 14000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will be bred as part of the project and checked shortly after birth for the presence of a human gene which causes disease. Some animals will be then taken forward for experimental work and some will be kept as breeding stock to generate new experimental animals. As we study diseases of the ageing brain, we often keep experimental animals to "old age", about 2-3 years in rat or mouse years. The studies we do are of two types. In some cases we age the animals over time to study behaviour or brain imaging before taking brain tissue to understand how a brain with a disease functions, or dysfunctions, differently from a healthy brain. We may inject substances, such as a disease protein, to see how that affects disease progression. In the second type of study we perform we treat the animals with new drugs to try to correct the deficits we have seen, for example by using a drug to remove the disease protein or protect the cells from dying.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Because we study diseases of the ageing brain, a proportion of the animals (about 1/3) will be allowed to grow old and develop symptoms reminiscent of a neurodegenerative disease of old-age, such as motor deficits and memory loss. Animals may also be treated with compounds to model the disease. Animals will experience the usual signs of ageing and some may develop difficulties with memory, gait balance and posture. Once we have identified disease-relevant changes in our mice and rats we will then test new therapeutics delivered orally, or injected into the body, or injected directly into the brain using surgery, to attempt to cure the disease. Animals will be very closely monitored following the onset of any symptoms and the disease will only be allowed to progress to a limited degree. All animals will be humanely killed 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 species)?

Overall, we expect 60% of the animals (rats and mice) used in this project to experience a Mild severity and 40% to experience a Moderate severity.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Diseases like Parkinson's disease and Alzheimer's disease affect how the whole brain functions and dysfunctions as the diseases develop. In order to be able to, first, understand how the mechanisms which drive the diseases to develop in the brain, and then to be able to test potential new therapies, we need to work with animals which show signs of the disease. We have chosen to work with mice and rats because of the shared similarity of the brains of rats and mice, as mammals, to humans, including the way brain cells are wired together into circuits, the way in which the brain is protected by a "blood brain barrier" and the way the immune systems changes as disease develops. We use genetically altered mice which develop early stage forms of the disease as this is the stage of disease we wish to study, and ultimately treat.

Although the use of less sentient animals (such as flies, fish or worms) can be informative in biology there are a number of advantages of using rodent models to study progressive neurodegenerative diseases. The most common risk factor by far for developing a neurodegenerative disease is age and therefore it is essential to use a model in which the development of the disease pathology can be studied over a prolonged period, ideally years. A second important consideration is the presence of important disease genes in the animal we work with. For example, we know that the gene for alpha- synuclein, a key pathological protein in Parkinson's, is not present in invertebrates, such as flies and worms. Finally, there is a very high degree of similarity between rodent and human genes and a conservation of structure, function and connectivity in the mammalian brain shared between rodents and humans. Overall, this makes rodents the best animals to model diseases of the brain in to understand the mechanisms and seek cures.

What was your strategy for searching for non-animal alternatives?

The animal work we do runs in parallel with other complementary approaches in our laboratory. First, we study diseases using neuronal cells cultured in dishes which is useful for generating preliminary data on disease processes. In particular, we work with patient-derived stem cells which can be used to generate different neurons of interest, such as dopaminergic, cortical and motor neurons. This cutting- edge stem-cell technology represents a powerful platform both to investigate mechanisms of disease and screen for novel molecular therapies which impact on cellular disease processes and may have



therapeutic value. Second, we study human post-mortem brain tissue donated by patients who have died from neurodegenerative disease. This more traditional approach remains very useful as it gives information on the real brains of patients who died with diseases such as Alzheimer's or Parkinson's.

Why were they not suitable?

Both the work in human stem cell-derived neurons and patient post-mortem tissue are very useful but have certain limitations. The work in cultured cells tells you how neurons may die but not how the brain as a "wired circuit" will respond to disease. Human patient post-mortem brain material is very valuable to study, but it does only represent the end-stage of the neurodegeneration, and provides limited information on the mechanism of disease progression over time. The human brain is an inaccessible and highly complex organ which makes it almost impossible to obtain patient "brain samples" during life. We therefore need to use animals in our project but only after we have obtained as much information as possible from those experiments which do not require 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 use animals for a range of experiments to study the clinical and pathological signs as the diseases develop. Previous experience and statistical power calculations allow us to define the number of animals we need in each experiment to observe alterations in behaviour, or to detect biochemical or pathological changes. Approximately 10-20 animals per strain are allowed to grow into old age for each experiment and to develop symptoms, which in rat and mouse terms is about 2-3 years old. We will test behaviour, co-ordination and memory of the ageing rodents, perform imaging studies, and finally, analyse the brain in detail when they die. By performing many tests on each animal, we will minimise the numbers of animals we need to use.

We use mice which have been genetically altered to develop an early stage of disease, such as Parkinson's or Alzheimer's. We breed ourselves all the mice which we need and most of the mice we use are required for breeding only and will not be allowed to age, or to develop any disease symptoms or used for testing therapies.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We take extensive statistical advice from experienced researchers in the field, combined with our own previous experience of working for over 15 years in the field, and statistical power calculations to define the number of animals we need in each experiment to observe alterations in behaviour, or to detect biochemical or pathological changes. Approximately 10-20 animals per strain are allowed to grow into old age for each experiment and to develop symptoms, which in rat and mouse terms is about 2-3 years old.



We are able to take multiple samples from one brain to be able to reduce numbers. For example, the brain has two halves which are essentially identical. So, for example, we are able to treat each half of the brain differently and collect different information and samples, allowing to collect more information and samples per animal.

What other measures apart from good experimental design will you use to minimise numbers?

We monitor very actively the breeding strategies we use which are all optimised for animal use. All breeding animals are followed individually and all schemes are recorded on a computer to follow animals pedigrees. Optimising the breeding numbers is important to ensure neither too few, or too many animals, are generated. Tissues are collected from animals and shared across project to make best use of all material. Pilot studies may be used to estimate the effect size of a drug treatment, or to confirm a drug is not toxic, before full-scale test are performed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

In our work we use mice and rats genetically altered to carry genes which, in humans, lead to developing a genetic form of diseases of the ageing brain, such as Alzheimer's or Parkinson's. We study the effect of disease genes on the brain of rats and mice to understand how the disease develops in human, and then test new ways of how to treat it. Mice and rats carrying disease genes are aged to mimic age-related diseases and monitored carefully over time. Our work aims to study and treat the very earliest forms of disease which also causes the least pain, suffering distress or lasting harm to the animals we use.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

As mammals like us, mice and rats conserve many of the brain structures found in humans affected by disease which we need to study and represent the simplest mammal we can work with. Other animals sometimes used in research, such as flies and worms do not have the same brain structure nor do they carry the same disease genes found in humans. We do use embryos or new-born pups to provide tissue to generate neurons to grow in the laboratory which provides a useful additional means to study the disease and also to test new drugs.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?



We have extensively refined the methods that we use to generate rodent models of neurodegenerative disease. Modern accurate genetic techniques act as a refinement and allow us to manipulate genes in rats and mice and express human disease genes in the right part of the brain at the right time to lead to disease processes and symptoms highly reminiscent of the human disease being studied. We focus on studying the early stages of disease because that is the key therapeutic window which we would want to treat patients in the clinic. As a result, we study and treat animals with generally only a mild form of disease. Animals are handled and trained carefully for behavioural tests to ensure the best most reliable and reproducible data are obtained. Animals are very closely monitored following the onset of any symptoms and the disease will only be allowed to progress to a limited degree.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow advances as publicised on the NC3R website which provides information on the most refined techniques, such as new guidelines on non-aversive methods of picking up mice, single-use of needles and blood sampling. We will adhere to updated ARRIVE guidelines on reporting work with animals as now required by many journals.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will follow advances as publicised on the NC3R website which provides information on the most refined techniques, such as new guidelines on non-aversive methods of picking up mice, single-use of needles and blood sampling. We will adhere to updated ARRIVE guidelines on reporting work with animals as now required by many journals.

Explain the choice of species and the related life stages

We will study strains of rats and mice carrying forms of human genes involved in neurodegenerative disease, such as Parkinson's, Alzheimer's and motor neuron disease. We use rats and mice as they have a similar brain structure to humans and share almost all the same genes. Most of the animals we will use will be young breeding animals used to maintain the strains. As neurodegenerative diseases generally affect old people a proportion of the animals (about 1/3) will therefore be allowed to grow old (which in rat and mouse terms is about 2-3 years old) and develop symptoms reminiscent of a neurodegenerative disease of old-age, such as motor deficits and memory loss.



159. Technical developments in imaging for in vivo application and use in cancer detection 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

No answer provided

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 is the aim of this project?

This license will allow us to continue our developments in imaging technology and to apply these to cancer imaging and therapy. In addition, it will allow us to make these new developments available to the wider imaging community as we will apply them to other disease areas, not just cancer.

Improvements in imaging technology will enhance measurements of disease, promote translation between animals and man, and contribute to the 3Rs in animal research.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Imaging is the method-of-choice for diagnosing, understanding, and monitoring a variety of disease. Since measurements can be non-invasive, the development of disease and the



effects of intervention can be assessed using a wide range of anatomic and functional measures in the same subject.

Imaging is a very powerful technique as it:

enables detailed, fast and accurate visualisation, characterisation, and quantification of biological processes in vivo

provides time-resolved, repeated measurements in the same subject resulting in improved analysis, increased experimental quality and great reductions in animal numbers.

can operate non-invasively, enabling measurements that cannot be achieved using more conventional methods such as histology or biopsy where tissues are removed for examination by a pathologist.

can simultaneously assess changes in both structure and function of organs and tissues, providing a more comprehensive characterisation of the disease.

can be used to plan and to measure the effects of therapeutic intervention and define windows-of- opportunity for successful treatment.

However, all imaging techniques require skilled interpretation with defined rules of reporting. Through development of new imaging techniques, optimisation of existing imaging techniques and validation against gold standards, we will:
minimise ambiguity in image interpretation and reduce the possibility of false positive/negative findings

lower the detection limits of disease

refine the imaging experience for the animal

Through the combination of imaging with therapeutic techniques, we will be able to deliver more accurate and precise therapies, and monitor disease progression and the response to treatment significantly better than before.

What outputs do you think you will see at the end of this project?

The outputs will include new and better animal handling apparatus, scanning methods and data analysis tools, with dissemination through presentation, publication and distribution of techniques to other groups and imaging centres.

The developments made will include: improved animal handling apparatus that is easier for the operator to use and decreasingly invasive or intrusive to the animal; faster and more sensitive scan modes that reduce the anaesthetic burden upon the animal and relate directly to Objectives 1 and 4. More comprehensive, increasingly clinically relevant and increasingly easily translatable studies that will advance treatment protocols in the clinic, most probably during the lifetime of this licence, result from Objectives 2, 3 and 4.

Many of the engineering developments resulting from these works will be applicable in a range of disease areas, for us most notably in cardiology and neurology as we have ongoing collaborations centred around the use of our developments in other centres' works.



Hardware and software developments will be described in the scientific literature and distributed to other groups, in some cases via inclusion in instrument manufacturers' products. Discoveries resulting from the increasingly well characterised disease progression are and will continue to be disseminated via the scientific meetings and literature and by inclusion in our disease-focussed collaborators' works.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The combination of less invasive animal handling apparatus and workflows, coupled with faster and more sensitive imaging techniques will both refine the animal experience and reduce the number of animals required for study. These benefits will be realised with each deployment of a better technique.

Faster and more robust scanning techniques reduce the cost of and training requirement for imaging, thus lowering the barrier to replacing conventional, invasive measurements such as histology. Proof-of-concept studies demonstrating this will be possible and will benefit those currently not practising imaging works in a range of disease areas. These benefits will be realised over the course of this project.

The demonstration of better imaging and collaboration with scanning manufacturers will help deploy some of the imaging developments on an international scale, again reducing the barriers-to-imaging and increasing uptake of this non-invasive technology. These benefits are realised on an ongoing basis as suppliers increase the range of functionality in their systems.

Finally, the availability of the improved measurement performance naturally leads to use of the incorporation of these technologies into imaging and biological research programs. More robust examinations of cancer progression and treatments will result and applications in disease areas unrelated to those we study are expected. These benefits are realised on an ongoing basis.

How will you maximise the outputs of your work?

Output from these works will be measured in terms of publishable output in journals of International repute (both by our group and our collaborators), by integration of our imaging developments into commercial imaging systems and by distribution of our techniques to other imaging groups.

Apart from publishing in internationally renowned peer-reviewed journals, we will also submit our reports on validation of the technology and scientific and welfare findings to scientific conferences held at national and international levels. Once validated, our new imaging developments, including image-guided therapies, will be handed over to our user groups to enable their research programmes which will further enable distribution our technologies.

Furthermore, we developed an extensive network of users for the imaging technology developed previously and we will continue doing so during this project. To aid dissemination of our knowledge to other groups, we are developing a website for facilitated distribution of the technical (software and hardware) aspects of our new developed imaging techniques. As such, future users can then assemble and deploy our technologies, ensuring sustainability of our technologies as it moves into more routine



usage and allowing for dissemination beyond the existing collaborative networks of the team.

Species and numbers of animals expected to be used

- Mice: 2000
- Rats: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

A staged approach will be used to develop new imaging techniques; methods are only tested *in vivo* once it is confirmed that the technique works in non-living systems, and methods using tumour models and/or recovery anaesthesia will only take place once basic *in vivo* demonstration has been achieved.

Basic *in vivo* demonstration typically consists of:

General anaesthesia using isoflurane in oxygen-enriched air for < 1 hour during which the following techniques will be performed:

Fluid replacement using subcutaneous injection of saline is used to ensure physiological stability

Placement (non-surgical) of a tail vein cannula for reliable compound delivery.

Placement of physiological monitoring apparatus to ensure animal welfare during imaging; a rectal temperature probe, subcutaneously-implanted ECG needles or topical ECG gel electrodes and/or topical respiration monitor will be used to monitor the animals' vital signs.

Intravenous dosing of contrast agents to improve the sensitivity of the imaging.

Imaging of anatomical structures or functional activity

Schedule 1 kill.

Advanced *in vivo* demonstration typically consists of:

A subcutaneous tumour is implanted whilst the mouse is under a brief period of general anaesthesia. Such tumours typically grow from palpable tumours at 1 week post inoculation) to 1000 mm³ tumours within 3-4 weeks.

Local, fractionated radiotherapy, replicating clinical practise is performed. Multiple low, rather than a single high, doses of radiation are delivered to the tumour on consecutive days, under general anaesthesia once the tumour reaches 100 mm³.



Imaging is then performed under general anaesthesia for <60 min per session, and repeated 3x/week for 3 weeks. During each anaesthetic session, the following techniques will be performed:

Fluid replacement using subcutaneous injection of saline is used to ensure physiological stability and aid recovery times

Placement (non-surgical) of a tail vein cannula for reliable compound delivery

Placement of physiological monitoring apparatus to ensure animal welfare during imaging; a rectal temperature probe, subcutaneously-implanted ECG needles or topical ECG gel electrodes, and/or topical respiration monitor will be used to monitor the animals' vital signs.

Intravenous dosing of contrast agents to improve the sensitivity of the imaging
Imaging of anatomical structures or functional activity

Additional procedures to be performed under terminal anaesthesia at the end point of the study:

Intraperitoneal dosing of histological markers for validation against a gold standard

Cardiac blood sampling for e.g. confirmation of the health status of the animal or for quality control of the contrast agent (e.g. metabolite concentrations)

Schedule 1 kill.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Tumour inoculation will only be performed under general anaesthesia which may induce transient pain at the site of injection, expected to be of short duration (hours).

Most animals will experience effects of gas anaesthetic as they recover. Good temperature control and rehydration protocols, including the availability of ingestible hydrogels post recovery is used to minimise this. Durations of anaesthesia and repeat intervals are set to minimise cumulative effects which, where they occur, would be of short duration (hours to days).

Transient pain may be expected consequent to the use of subcutaneously, intraperitoneally, intramuscularly and intravenously positioned needles for dosing, and for subcutaneously implanted needles for ECG measurement. These are positioned and removed whilst the animal is under general anaesthesia and the effects, where they occur, are likely to be of short duration (hours). Where intramuscular dosing is performed analgesia will be applied, and where any behaviours indicating pain are present analgesic and/or emollient cream will be applied.

Following radio-, photodynamic and ultrasonic therapies transient wounding to the skin may occur. In this case analgesic cream will be applied and emollients may be applied. Where the skin is broken it must repair within 24 hours, where the skin is bruised it must be repairing within 72 hours or the animal will advance to Schedule 1.



Food deprivation, as required for some PET imaging, may induce a transient weight loss, and will last no more than 6 days. Weight loss may also be a result of cumulative exposure to anaesthesia and/or treatment, and is expected to be of short duration (days).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

For both mice and rats we expect:

Non-recovery: 50%

Mild: 20%

Moderate: 30%

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 live animal is required because many of the phenomena and features we need to image only exist in the living body. Some of these, such as tissue volume or constitution, are the target of measurement whilst some of them, such as the involuntary muscle jerks, cardiac and respiratory cycles and peristalsis, are confounds which corrupt the imaging process. We aim to develop imaging techniques that are insensitive to these confounds whilst being maximally sensitive to the measurements required.

Our primary disease focus is cancer. Some aspects of tumour development is currently too complicated a task for computational or cell-culture biology to model at the whole-body level as the development of tumour requires host-tumour interactions that only occur in the intact living body.

What was your strategy for searching for non-animal alternatives?

Basic and prototype developments are performed using computer, mechanical and electronic simulations, with progression to in vivo taking place only after successful demonstration of the development in a direct pre-plicate of the in vivo experiment.

Why were they not suitable?

As this licence is specifically aimed at technology development that will benefit others who have already committed to in vivo experimentation we need to apply and validate the resulting technology in the live animal before making the said technology available to the community.



Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 gained extensive experience through the use of our predecessor PPLs and we have used this past experience to estimate our numbers of animals for this project.

Most of our statistical analysis is very straightforward as we tend to be examining a binary question; is the new imaging technique better than an existing technique (*yes or no*)? Typically, small sample sizes and numbers of animals (often <20 per development) satisfy this need. Validation may require a further 20 mice and ongoing quality controls over the term of the project double this.

Using this approach we can expect to complete >20 developments for mouse and 1 development for rat over the term of this project.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We will minimise the number of animals required by developing techniques using test objects, computer, mechanical and electronic simulations wherever possible, and only advancing to *in vivo* once techniques are demonstrated to work. Demonstration of techniques (as opposed to, say, evaluation of drug efficacy) usually requires small numbers of animals as the measures of success tend to be binary (does the technique work or not). For more complicated questions, we will liaise with the the Statistics Unit for advice on statistical experiment design and for performing power calculations to obtain meaningful data.

It is well established that repeated *in vivo* imaging allows a significant reduction in the sample size required to achieve a particular level of statistical significance.

The aim of this project is to develop techniques that enable better measurements of disease progression and response to treatment, using imaging techniques. These improvements may offer better imaging data, better imaging data collection efficiency, and/or better animal welfare compliance. All three of these provide opportunities for minimising the number of animals required.

Tools such as the NC3Rs' Experimental Design Assistant will be used wherever possible and we will make every attempt to incorporate the ARRIVE guidelines into the design of our studies.

What other measures apart from good experimental design will you use to minimise numbers?

Methods are only tested *in vivo* once confirmation that the underlying technique works in a preplicate simulation of an *in vivo* experiment.



Works in tumour models and/or with recovery will only take place once the techniques work in naive animals and where the tumour or recovery is required for the validation.

We will compare our newly-developed methods with existing techniques and quantify the benefits with additional functionality introduced in a stepwise fashion. Developments stop, and methods are made available to the community, as soon as the technique is validated.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mouse and rat are chosen as these are industry standard for use in preclinical imaging development works. The rat will only be used where the physical size of the animal to be imaged or treated dictates.

All imaging techniques and related measurements will be developed, evaluated and validated in stages so we can control as many variables as possible at the same time to make technique development as efficient and successful as possible. This approach is also reflected in the use of the different animal models.

During the initial stages of development, no disease will be introduced and all procedures will be performed under terminal anaesthesia during which the animal remains insentient. Only when the new development works for the normal animal without causing any welfare issues, we will proceed with models of disease and/or with recovery anaesthesia.

Superficial xenografts will be considered for the next stage of the development pipeline. They offer us a simple means to introduce the disease variables whilst we still control the growth kinetics and the location of the tumour. Tumours will be generated by injecting tumour cells subcutaneously under a very brief period of general anaesthesia. We may implant cells in up to two sites in order to enable us to use one tumour as the internal control for the other. We typically co-inject the cells with materials to promote tumour growth; for example the basement membrane preparation Matrigel. The tumour remains localised to the site of injection and no metastasis occurs. Therefore the animals do not generally suffer adverse effects. We employ humane euthanasia before combined tumour volume reaches 250 mm³ (mammary fat pad) or 900 mm³ (s.c. xenografts) to avoid any impairment of mobility. Measuring superficial tumours is conducted in conscious animals using callipers or will be image-derived.

It is sometimes needed to use more complex models of disease as differences in tumour heterogeneity, perfusion, microenvironment and host tissue involvement will affect the image presentation and/or measurement. In these cases we need to generate the tumour within the organ of interest; breast, lung or pancreas cancer cells grown in the mammary fat pad, lung or pancreas, respectively. Injection into the mammary fat pad or lungs will typically take place under a brief period of general anaesthesia, in a similar manner to the generation of subcutaneous tumours. As with the subcutaneous models, we may implant cells with biomaterials such as Matrigel to promote growth or to make sure the cells stay in



the intended site (percutaneous lung model). Currently, orthotopic pancreas tumours require abdominal surgery, conducted in accordance with the LASA 'Guiding Principles for Preparing for and Undertaking Aseptic Surgery'. Surgeries will be carried out early in the working day to allow sufficient time for assessment of animal recovery prior to the overnight period. Animals will be given pre- and post-operative analgesia, and replacement fluids. The tumour cells will be co-injected into the tail of the pancreas (as opposed to the head) with matrigel in order to limit adverse effects and improve chances of successful tumour growth. The humane endpoints relating to the post-surgery period and the burden of pancreatic tumour growth are fully detailed in the relevant protocols. We will employ humane euthanasia if the pancreatic tumour reaches 900 mm³. There have been some publications in the scientific literature relating to ultrasound guided injection of tumour cells into the pancreas and this is being trialled by other groups in the department. This would represent a significant refinement to the animal. Seen our extensive expertise in imaging technique development, we will further develop this technique as a suitable alternative to abdominal surgery.

A substantial part of our planned work is the development of improved imaging techniques for the early- detection of lung metastasis. A similar staged approach will be followed; the percutaneous model, described above, will be used as the subcutaneous xenograft alternative for the lung. Although a carcinogen (urethane) will be used to induce the autochthonous lung model, the doses used are non-toxic and the tumours are slow growing with pre-determined growth kinetics. We will perform imaging to determine the tumour volume and employ humane euthanasia at an overall tumour volume of 200 mm³.

Wherever possible substances will be administered as isotonic aqueous solutions and in the smallest volume that can be accurately/safely administered. We will adhere to the limits on number and frequency of doses set out in the Protocols. Injections may be administered to conscious mice that are competently restrained, or to animals under gaseous anaesthesia. The intravenous cannulation route will be used where possible, in order to reduce the number of interventions the animal is subjected to and to make sure the injection route is clear. Oral administrations will be made to conscious restrained animals where possible, and formulations will be flavoured or sweetened where possible to render them palatable to the animals. In occasional cases we will administer injections directly to the tumour. Drugs will be administered in doses and formulations shown to be non-toxic and well tolerated; either from the experience of colleagues or collaborators or the scientific literature.

Radiation beams, photodynamic treatments and biomedical ultrasonics will be applied to tissues and tumours. This will be conducted under gaseous anaesthesia. Radiation dose to regions other than the target is minimised either by shielding with lead or the use of a focused image-guided micro-irradiator.

From experience we know that a single dose not exceeding 30 Gy or fractionated dosing (up to 5 Gy/day, and not exceeding a total dose of 50 Gy) is well tolerated. For irradiation of orthotopic pancreatic and lung tumours, igRT will be used. PDT treatment consists of a single dose of PDT-drug at non-toxic concentrations prior to a non-thermal illumination light dose. Damage to non-target tissue and surrounding skin is minimised by the use of small diameter laser beams and precise positioning of the laser beam. Biomedical ultrasound therapeutic delivery protocols will only be used when optimised by other research groups. Humane endpoints for any therapeutic delivery are stated in the Protocols and humane euthanasia will be applied when desquamation of the skin, reddened skin, weeping skin or ulcer development occurs over an area larger than 110% of the treated target area.



Anaesthesia is often required for subject immobilisation during imaging developments. Animal physiology will be monitored by core temperature (e.g. subcutaneous or rectal temperature probe), respiration rate (e.g. pneumatic or piezoelectric detector) or ECG (e.g. subcutaneous electrodes). Temperature will be maintained using a thermostatically controlled heating pad. For non-terminal anaesthesia, animals will be monitored until they have fully recovered from general anaesthesia. We are developing alternatives for non-invasive ECG monitoring. The duration and repetition rates of anaesthesia are set to allow measurement of parameters of interest whilst maintaining adequate health and welfare status of the animals.

For extended durations of anaesthesia (>200 minutes), a 72 hour minimum recovery period will be invoked and confirmation (by NACWO) that the animal is sound will be required before repeating anaesthesia.

In a limited number of cases where the aim of this work is to allow examination of the time-dependent processes/alterations in for example vascular function, then highly-repeated anaesthesia will be performed. If so, the repate rates will be limited to 10 times in 20 days with a maximum duration of 90 minutes per session.

We seek to develop minimally invasive techniques that can be applied without introducing significant harm to the animals and without altering the progression of the disease to be studied. The use of image-guided and image-monitored therapy offers the opportunity to maximise on-target delivery of treatment whilst minimising stray tissue damage. This along with the high-resolution and fast imaging that is available to us enables highly efficient deployment of these techniques for use in other research programmes.

The most invasive techniques will be performed under terminal conditions during which the animal will remain insentient. Protocol 4 is restricted to procedures to be performed under terminal anaesthesia to allow translation of our methods to other disease models so the wider scientific community can benefit from our most refined imaging techniques.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mouse and rat are chosen as they are the industry standard for use in preclinical imaging development works and methods will be, as far as is possible, scalable to use in larger species and man. Dedicated small animal imaging systems, that replicate those in clinical practice, have been optimised for use in mice and rats, the species most abundantly used for the study of human disease.

Moreover, the mouse is the most studied animal model in the field of oncology and other disease areas, and therefore there is a wealth of literature available to guide experimental design. This is mainly because the mouse shares similarities to humans that do not exist in lower species, and they develop tumours as a result of mutations in genes known to cause cancer in humans.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?



We seek to develop minimally invasive techniques that can be applied without inducing significant harm to the animal and without altering the progression of the disease to be studied.

We operate programs of developments in animal handling apparatus, physiological signal monitoring, scan control and system integration methods. This forces a highly structured and standardised approach to in vivo imaging with standardisation aimed at the highest level of performance. As a direct result of this holistic approach animals are physiologically monitored using minimally invasive techniques and imaged with the shortest possible scan times. Minimum necessary anaesthetic durations are, therefore short.

Application of the techniques developed offer scope for significant refinements especially for use under the authority of other biologically-oriented PPLs. MR-image guided radiotherapy (MR-igRT), as previously developed by our group, allows a directly clinically translatable technology to be used with a level of robust performance that is scientifically sound, economical and highly refined. With MR-igRT, abdominal tumours are accurately and precisely treated during short anaesthetic periods that are repeated over several days. This protocol, favoured in the clinic, maximises treatment of the tumour whilst minimising side effects elsewhere, thus providing a major refinement.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Experiments will be based on the Guidelines for the welfare and use of animals in cancer research (Workman P et al. Br J Cancer 2010, 102: 1555-1577).

Recommendations from NC3Rs will be followed where possible; e.g. tunnel handling, single use of needles.

In addition, we will keep up to date with the literature and implement new recommendations as they are published; e.g. FASEB J. 2019 Mar; 33(3): 3097–3111.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We are already liaising with the NC3Rs regional officer. NC3Rs news letters (both at local level through the REDACTED and at national level through NC3Rs) will be consulted.

Furthermore, we routinely attend and present at animal welfare conferences and symposia such as IAT, NC3Rs, American Association for Laboratory Animal Science - AALAS.

Explain the choice of species and the related life stages

Adult mouse and rat and are chosen as these are industry standard for use in preclinical imaging development works, and rat will only be used where the physical size of the animal to be imaged or treated dictates.

The mouse is also chosen because of availability of genetically altered mice which often provide the best mechanistic model of various pathological mechanisms. Well-characterised, genetic alterations to animals will be used where these are essential for the generation of image contrast or where the development of pathology requires this.



160. The aetiology of diabetic neuropathy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.

Key words

Diabetes, neuropathy, nerve, regeneration, sensory

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim for this programme of work is understand the mechanisms underlying the key functional changes that take place in the nervous system in diabetes and develop treatment strategies to reverse or prevent these 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.

What are the potential benefits that will derive from this project?

Diabetes mellitus affects 9% of the population (an estimated 463 million people worldwide) and the incidence is ever increasing. Approximately 50% of people with type 1 or type 2 diabetes will develop some degree of diabetic neuropathy (damage to nerves) which may be accompanied by tingling and burning sensations, heightened sensitivity, or numbness and loss of sensation. This can lead to tissue damage, infection and an increased risk of limb amputation.

Diabetic neuropathy can also affect internal organs, for example causing gut and bladder problems. It is a debilitating condition which impacts on patient's health and quality of life. There is currently no treatment for diabetic neuropathy, other than managing blood sugar levels or treating symptoms, and this is an urgent unmet clinical need.



From our studies we hope a greater understanding of the mechanisms underlying diabetic neuropathy will enable a realistic prospect of development of novel targeted therapies.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

We use rodent (rat/mouse) models of diabetes and have estimated 1500 animals over a 5 year period.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

We use rodent models of Type 1 and Type 2 diabetes to study the development and progression of diabetic neuropathy. With both models, animals will develop raised blood sugar levels, eat and drink more and urinate more frequently than non-diabetic rodents. Animals are regularly checked and their condition monitored. Cages are changed daily, and animals have unlimited access to drinking water and food, which are provided in extra amounts. Animals with Type 1 diabetes do lose weight, so weights and condition are carefully monitored. The severity of diabetes is controlled by implantation of a low-dose insulin delivery pellet under the skin (conducted under anaesthesia and with post-operative analgesia) this ensures that animals develop raised blood glucose (at levels that might be seen in poorly-controlled clinical diabetes) and neuropathy, but prevents morbidity due to the diabetes. Animals with Type 2 diabetes, become obese and may develop skin irritation, again conditions and weights are carefully monitored. We assess sensory, motor and cognitive function over the timecourse of the study using tests that do not cause fear, pain discomfort or any tissue damage, and do not expect any adverse effects from any tests selected.

Then, at the end of the study, animals are terminally anaesthetised to assess nerve conduction speeds and bladder function. Animals are euthanased at the end of the protocol and tissues collected for molecular and biochemical analysis

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

In our laboratory we routinely use cell culture to both complement and help us design our in vivo work. However diabetes is a whole body disease, affecting the hormonal, immune, cardiovascular and nervous systems which all play a role in progression of the disease, and it is impossible to wholly mimic in a culture dish. The complications of diabetes are progressive and take time to develop and are the product of several consequences of poorly controlled diabetes. Hence there is a need for animal models of diabetes. The



similarities between diabetes in humans, mice and rats justifies the use of animal models of diabetes to elucidate the mechanisms of the disease process, in characterising the changes in the nervous system and assessing the efficacy of therapeutic agents. If any relevant non-animal alternatives become available during the course of the project, we will incorporate these in our studies.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Experiments are designed on the basis of previous work and published data and we refer to the ARRIVE guidelines. Full evaluation of previous/pilot data and power calculations are performed, such that the minimum number of animals required to provide valid data are used (typically 14 per group for therapeutic studies).

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The animal of choice for this work is a lower order mammal, typically the rat, although mice may also be used. We typically use the streptozotocin Type I model of diabetes and are familiar with its advantages and limitations as well as the specific requirements for animal welfare. Our results can be integrated with the continuously expanding body of research conducted on these species and model, and contribute to comprehensive understanding of the pathogenesis of diabetic neuropathy.

Numerous strategies are in place to minimize animal suffering, we use appropriate anaesthetics and analgesia for any surgery. Sensory and cognitive testing do not cause tissue damage or pain and animals are handled and acclimatised to minimise any undue stress, and we study innate behaviours.



161. The benefits of sleep and the cost of wakefulness

Project duration

5 years 0 months

Project purpose

None selected

Key words

Sleep, wakefulness, brain, sensory stimulation, neurodevelopment

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The project aims at improving our knowledge about the functions of sleep. It centres on three interrelated questions: 1) How sleep is regulated? 2) What are the consequences of inadequate sleep? 3) Can we take advantage of the beneficial functions of sleep to improve our health?

Sleep is a complex behaviour and is tightly regulated. We have all experienced that undeniable drive to sleep. Staying up much later than usual or rising after only a few hours of sleep and then attempting to stay alert and functional throughout the day, serve as unpleasant reminders of the power of sleep drive. Even when we feel alert and are unaware of our sleep drive, it is always present and growing while we are awake. In fact, the only true way to reduce rather than mask sleep drive is to sleep. What is the sleep drive made of and what are the mechanisms regulating it? These questions will be addressed by quantifying the cellular changes in the brain in conditions with high sleep drive (e.g. after a long period of wake) and low sleep drive (e.g. after a long period of sleep).

In modern society, chronic insufficient sleep (defined as gaining less sleep than required) has become epidemic worldwide, in particular among adolescents, and represents a serious health risk. Insufficient sleep is associated with higher risk of developing neuropsychiatric and behavioural disorders, such as anxiety and depression, that are the primary drivers of disability worldwide. This project will help clarify the consequences of sleep loss by measuring the short and long-term responses of brain cells to insufficient sleep. By gaining insight into the underlying biological mechanisms linking chronic insufficient sleep to altered brain function I aim to identify potential therapeutic targets to improve mental health.



Recent research has demonstrated that boosting some cardinal features of sleep (e.g. sleep intensity and efficiency) is possible by delivering sensory stimulation during sleep. Both acoustic and vestibular (i.e. rocking) stimuli have been effective in enhancing sleep. In this project, I will apply this approach to rodents to understand whether some brain functions can be promoted by enhancing sleep. This research will help clarify why sleep is beneficial to our health and whether sleep can be manipulated to enhance the capability of our brain to perform better, particularly in the pathological context of debilitating neuropsychiatric diseases, such as Alzheimer's.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

The proposed project will improve our understanding of the mechanisms and functions of sleep. It will shed light on the detrimental consequences of sleep loss on cognition and mental health, in particular during sensitive periods of brain development. Finally, it will clarify the effects of sleep enhancement on brain functioning.

The acquired knowledge will benefit other scientists (e.g. clinical researchers in neurology and clinical sleep scientists) in the short term and clinicians in the medium/long-term, in light of the prevalence of sleep disorders in modern society (20-30% of adults report sleep problems). Finally, a better understanding of the impact of insufficient sleep will help inform society and policy makers of the risks posed by chronic sleep deprivation.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Approximately 2320 mice and 910 rats over 5 years.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The kind of investigations that are proposed require healthy rodents. Therefore, housing and handling conditions are designed to optimise welfare and any surgical procedures make use of full anaesthesia, health monitoring and analgesic regimens recommended by veterinarians. In the sleep deprivation/restriction experiments, rodents will experience tiredness and desire to sleep. Experiments are designed in such a way that animals will never experience physical exhaustion due to these procedures. Animals may sometimes have controlled access to food to motivate task performance, but they will be given highly palatable and nutritious rewards during behavioural tests that tap into rodents' natural preferences (e.g. exploring mazes, poking their noses in holes). This range of experiments



will cause mild to moderate discomfort. Once experiments are complete, animals will be humanely killed, and brain tissue collected to support further data analyses.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Sleep is a behavioural state and as such it can only

be defined and investigated in animals. The consequences of sleep deprivation involve the entire organism and can only be studied in animals. In order to study sleep as a global phenomenon, there are no alternatives but using a freely behaving animal. Non-animal alternatives such as in vitro or computer models do not replicate the complex neuronal mechanisms that underlie sleep and its manifold disturbances.

Humans cannot be used to study changes at the circuit or cellular level due to the invasive procedures necessary, as well as the inability to collect tissue.

Reduction

Explain how you will assure the use of minimum numbers of animals.

When possible, a within-subject design will be adopted, meaning that the number of individual rats and mice used is kept to the absolute minimum required to allow statistically robust conclusions.

Experiments will be planned carefully to optimise data collection by addressing multiple objectives during a single procedure (for example by performing histological analysis in the same animals used for behavioural/electrophysiological experiments).

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The rodent (rats or mice) has been chosen as the experimental animal because the physiological changes during the 24-hour waking cycle and the response to forced waking induced by sleep deprivation have been extensively studied in this species. However, the transgenic approach needed to visualize or manipulate the activity of individual brain cells is currently available mostly in mice. Therefore, mice will be preferentially used for these experiments. Rats, on the other hand, have more complex behaviour and bigger brains, therefore will be preferred to investigate the effects of sleep loss on behavioural tasks.





162. Redacted

Project duration

5 years 0 months

Project purpose

Basic research

Key words

No answer provided

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 is the aim of this project?

Using mice as a model, the goal of this project is to investigate if the supplementation of resveratrol or vitamin D can be used as effective nutritional strategies to treat and prevent the decline in skeletal muscle contractile function induced by the consumption of a high fat diet (HFD).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Literature in recent years has established that high calorific diets and more specifically adipose tissue accumulation, will negatively influence contractile function of skeletal muscle. Despite this, there has been very little literature on the reversibility on such negative effects. The limited research which has considered such has reported that dietary intervention alone is insufficient in reversing the decline in muscle function brought about by a high fat diet.

An emerging body of literature has begun to examine the possible anti-obesogenic effects of supplements resveratrol and vitamin D. Whilst both have displayed to reduce adiposity accumulation when given as part of a high fat diet. Current literature has not considered the therapeutic effects of vitamin D and resveratrol supplementation on contractile function



when used in conjunction with or following a high fat diet. As such, the current work will uniquely examine if supplementation of vitamin D and resveratrol can be used as a therapeutic strategy to reverse or prevent a decline in muscle function associated with consumption of a high fat diet

As the prevalence of obesity has quickly become a global epidemic, effecting upward of 30% of adults in the United Kingdom, which is ultimately costing the National Health Service an estimated £6.1 billion per year, it is important to begin to establish possible therapeutic methods to mitigate such declines in the contractile function of skeletal muscle. Without such scientific research a negative obesity cycle may continue, whereby poor contractile performance and inhibited muscle function lead to greater inactivity and subsequent weight gain, which exacerbate the declines in contractile performance. Undertaking this work will help progress current scientific knowledge on the therapeutic effects of both resveratrol and vitamin D on the contractile performance of isolated muscle and potential anti- obesogenic effects. Furthermore, this work may be used as a starting point in establishing the direct effects of the chosen supplements before progressing into a human model

What outputs do you think you will see at the end of this project?

We hope to identify possible avenues to treat or prevent a decline in contractile function associated with consumption of a high fat diet with resveratrol and vitamin D. Following completion of the study we hope to submit this work for publication (e.g., the International Journal of Obesity, or similar) and present the findings at conferences (e.g. European Congress on Obesity, or similar). The findings from the study will also be included as part of a Doctor of Philosophy thesis. If, as hypothesised, supplementation of nutritional supplements alleviates detrimental effects of a high fat diet on contractile performance of skeletal muscle, this work will used as part of future grant funding applications to further scientific knowledge on the chosen supplements and development into a human model.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short term the proposed study will be the first step in establishing the direct effect of resveratrol and vitamin D on muscle function, and understanding if the proposed strategies have a direct effect on the intrinsic force producing capacity of skeletal muscle. This work is not only important in developing our scientific understanding of the effects of both resveratrol and vitamin D short term, but it may also be a foundation for longer term future studies to implement nutritional strategies in a human population in order to negate the detrimental effects of obesity on muscle function. Obesity is at epidemic proportions globally, substantially impacting on health, reducing quality of life and is strongly related to mortality.

Obesity also has substantial financial implications for health care providers, costing the UK's National Health Service upward of £6.1B per year. Therefore, the current study may help in the development of future therapeutic strategies for weight management, which subsequently helps in reducing obesity related costs to health care providers.

How will you maximise the outputs of your work?

We hope to maximise the outputs from this work by submitting for publication and presenting the work at national and international conferences, irrespective of the success



of the nutritional strategy. By submitting for publication we further hope to develop the scientific understanding of the effects of both resveratrol and vitamin D. Furthermore, it may also be a foundation for future studies to implement nutritional strategies in a human population in order to negate the detrimental effects of obesity on muscle function. The study may also lead to future research grant funding, probably with existing or new external collaborators, as we develop a plan to extend this work based on these findings. Alternatively, if there were to be no effects of selected nutritional supplements on the contractile performance, publishing such results will still influence future work as they may consider alternative doses, durations or different nutritional supplementation before progressing into a human population.

To ensure effective and rigorous reporting of our results, we shall write papers according to the ARRIVE guidelines which are recognised as providing excellent transparent standards for reporting of research using animals and were developed by the NC3Rs.

Species and numbers of animals expected to be used

- Mice: 840

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mice will be fed one of the following altered diets ad libitum for up to 16 weeks;
High fat diet (e.g 60%Kcal fat)
Standard laboratory diet

Resveratrol or vitamin D enriched High fat diet
Resveratrol or vitamin D enriched standard diet
Mice will be schedule 1 culled at the end of the 16 week altered feeding regimen

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The nutritional supplements have no previously reported adverse effects. The use of the supplements is to promote positive effects such as decreased weight and fat gain and mitigate the decline in muscle function normally seen in high fat diets.

Whilst unlikely, if the animal finds their altered diet unpalatable they will be removed from the study. Weight loss/gain will be monitored regularly to ensure each animal is sufficiently nourished.

In the high fat diet groups a difference in body mass of up to 31% has been reported in groups who receive a high fat diet when compared to animals who receive standard lab chow. This work and other literature which has reported a similar increase in body mass in mice have reported no adverse effects for the animals. As such, we aim to evoke a similar change in body mass in a maximum of 16 weeks. However we expect this cut off point to



be achieved at an earlier point as previous work has indicated such changes occur between 12 and 16 weeks. Animals will be regularly monitored by trained staff and veterinarians using an AWERB approved score sheet (weekly weight and health monitoring) to establish humane end points for the animals e.g. weight gain is affecting cage density thus effecting the ability of the animals within the cage to ambulate or is causing pain/distress to the animal. In the event an animal is in pain or distress or find the diet unpalatable they will be immediately removed from the study.

Expected severity categories and the proportion of animals in each category, per species. What are the expected severities and the proportion of animals in each category (per species)?

approximately 80% animals will experience mild severities as the only changes the animal will experience is a change in diet. The remaining animals will receive a standard laboratory diets, as such, no severities are expected.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Whilst there has been an increase in human studies examining the effects of obesity on muscle function, an isolated animal muscle model offers a direct approach to assess the contractile performance of skeletal muscle, as outlined in a recent review article. Using relatively small isolated muscles allows for a greater understanding of the direct effect of obesity and nutritional strategies on whole muscle function and muscle quality. This form of assessment allows for tighter control of potential confounding variables related to the environment the animal lives in, including diet. Previous work which has isolated non-human muscle to establish effects of a HFD on contractile performance have identified muscle, fibre type and contractile specific responses. As such, isolated muscle is extremely valuable in understanding these specific responses and changes which may be brought about by supplementation of resveratrol and vitamin D during consumption of a HFD. There are also many variables during in vivo testing, such as adherence to dietary changes or ability to produce maximal contractions, which limit our ability to understand the direct effects of nutritional supplements on muscle function. In vitro testing is also a valuable first step in establishing direct responses of treatments, which has the potential to be the foundation for future studies which look to use such treatments in a human model.

What was your strategy for searching for non-animal alternatives?

Due to the current knowledge in the area of nutritional supplementation as a method to prevent or alleviate the effects of a high fat diet, non-animal alternatives cannot currently provide comparable detailed and robust information on the effects of such supplements on muscle function.

Why were they not suitable?



There are many confounding variables of an in vivo experimental design which will substantially impact the outcome of our understanding of nutritional interventions on muscle function. For example adherence to dietary changes and ability or willingness of participants to perform maximal contractions will limit our ability to accurately identify the effects of the chosen supplements on the function of skeletal muscle. Furthermore, in vivo experiments limit our ability to understand the direct effects of supplementation on muscle quality (force per unit of muscle) due to difficulties in isolating human muscle. Using isolated muscle allows for a greater understanding of the direct effect of obesity and nutritional strategies on muscle function and muscle quality. This form of assessment allows for tighter control of potential confounding variables related to the environment the animal lives in, including diet. As such in vitro testing can be used as a valuable first step in establishing direct responses of treatments and can be the foundation for future studies which transition into a human model.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Ten years of experience for the lead researcher in this field has shown that 10 animals per experimental group provides sufficient statistical power in studies of this nature.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The study design is cross sectional manipulation as we are looking at the same population (female mice, age matched) with modified diet. Where possible, factorial experimental designs will be used (i.e. each unique diet will be tested against each other possible condition) followed by appropriate statistical analysis (e.g. ANOVA, MANOVA, etc.). This minimises the number of animals that would otherwise be required if simple paired comparisons were used instead. Thus, the same statistical power can be achieved using the fewest number of animals.

What other measures apart from good experimental design will you use to minimise numbers?

We will minimise the number of animals required by electing to harvest two muscles per animal. Previous work has required a greater number of animals as they have often only harvest one muscle per animal. However, we are reducing the number of animals needed by half and we are confident that this is achievable based on other published work where we have used two muscles from an 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Female mice will be used, similar to previously published literature from our laboratory, allowing us to make direct comparisons between previous work, which will increase the outputs from the work without the need to sacrifice a greater number of animals. Furthermore, using female mice only ensures that sex does not cause any variability in responses to treatments.

As stated previously we expect there to be no harm toward the animal as they will only receive a modified diet, opposed to alternative methods which may induce stress through handling i.e. oral gavage. The nutritional supplements provided have not only reported no adverse effects, they have also shown to evoke positive responses such as improved contractile function and potential anti-obesogenic effects. In the unlikely circumstance they find the modified diet unpalatable they will be removed from the study. Animals will also be regularly monitored by trained staff and a veterinarian, where an AWERB approved score sheet will be used to ensure welfare of the animals throughout.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The life stage of the animals is used to represent physiological maturity and peak muscle performance. The treatment is of mild severity and should therefore cause non or limited pain or distress. Long term anaesthesia during dietary treatment would be impractical and unnecessary.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Animals will be regularly monitored i.e. weight gain, by trained staff and veterinarians to ensure animal welfare. If the animal displays signs of discomfort, malnutrition or weight gain is affecting cage density, they will be immediately removed from the study. An AWERB approved score sheet will be used for weekly weights and health monitoring checks.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The ARRIVE guidelines will be followed to ensure scientific rigor of the research, to maximise the research outputs and minimise and reduce the number of animals used and the severity they will experience. Furthermore, the most refined methodology and protocols will be taken from reputable journals and research groups which have experience in high fat diets, dietary nutritional supplementation and measuring contractile performance of isolated skeletal muscle in rodent models.

How will you ensure you continue to use the most refined methods during the lifetime of this project?



Explain the choice of species and the related life stages

Female mice will be used, similar to previously published literature from our laboratory, allowing us to make direct comparisons between previous work, which will increase the outputs from the work without the need to sacrifice a greater number of animals. Furthermore, using female mice only ensures that sex does not cause any variability in responses to treatments.

The life stage of the animals is used to represent physiological maturity and peak muscle performance. The treatment is of mild severity and should therefore cause non or limited pain or distress. Long term anaesthesia during dietary treatment would be impractical and unnecessary.



163. The efficacy of HCN2 channel blocking drugs in treating tinnitus

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

No answer provided

Animal types	Life stages
Guinea pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The aim is to obtain evidence that blocking HCN2 channels with specific pharmacological agents in our guinea pig models of tinnitus will lead to a significant reduction in tinnitus related symptoms.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is estimated that over four million prescriptions a year (in the USA and Europe) are for drugs to treat problems associated with the presence of tinnitus, such as anxiety, stress and sleeplessness. Tinnitus generally becomes much more noticeable and potentially annoying when experienced in a quiet bedroom at night. This may make it more difficult to get to sleep and can produce disturbed sleep and increased levels of stress and anxiety. Currently there are no pharmacologically effective treatments for the symptom of tinnitus itself, and no drug has been approved for its treatment (Langguth et al., 2019; Ann Rev



Pharmacol Toxicol 59:291-313). The current healthcare bill, for treating the problems associated with tinnitus on the NHS, is estimated to be 750 million pounds per year (Stockdale et al., 2017; BMC Health Services Research 17:577). Thus there is a huge market for a drug to reduce the tinnitus percept directly (Elgoyhen and Languth, 2011). Tinnitus is a symptom produced by a wide variety of aetiologies and there is clear evidence that more than one mechanism underlies tinnitus.

Fundamentally we do not fully understand tinnitus and have no broadly effective way to treat it (McFerran et al., 2019; Front. Neurosci. 13:802. doi: 10.3389/fnins.2019.00802 . We are hoping to identify a treatment, involving an oral medication, which would be easy to prescribe and of potentially wide use probably for the form of tinnitus that is comorbid with hearing impairment.

What outputs do you think you will see at the end of this project?

The drugs we will be using in this project have been produced by a commercial company as experimental drugs to try and target HCN2 receptors in different body systems such as the cardiovascular. The drugs have already undergone extensive testing in these systems and REDACTED is now interested in potentially repurposing them if we can obtain reliable evidence that one or both of them are effective in our guinea pig model of tinnitus. The drugs are nearing the end of their patent protection and we do not expect any delay being placed on the publication of papers based on our data.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The drug company will receive information about whether or not there is evidence that these particular drugs may be effective against tinnitus. This will also be of more general use because other more specific HCN2 blocking drugs, that have higher binding constants, have recently been developed and are awaiting patent protection. If our less-specific drugs are effective against tinnitus then these new drugs are also likely to be effective against tinnitus. By using the older drugs we will be able to publish our results and thereby update other scientists about our results. Many previous attempts have been made to find a drug that is effective against tinnitus but these have all been unsuccessful. If we can obtain positive results and publish them then this will encourage other groups to investigate this class of drugs for treating tinnitus. This should speed up the identification of a drug that can treat tinnitus even if these two particular drugs are not considered to be a sufficient solution.

How will you maximise the outputs of your work?

This work involves collaboration with a drug company. Obtaining reliable results about the effectiveness of these drugs against tinnitus should encourage REDACTED to start to develop these or even more effective drugs as a treatment for tinnitus. As the two drugs are nearing the end of their patent protection there is no bar to publishing the results in high impact journals and presenting the results at international meetings and this is what we intend to do. We will also present the results to patient's organizations such as the British Tinnitus Association.

Species and numbers of animals expected to be used

- Guinea pigs: 130



Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

We have two models of tinnitus:

is a rapid onset, reversible form of acute tinnitus that involves the subcutaneous (s.c.) injection of a high dose of sodium salicylate (the active form of aspirin). This form occurs in human patients who receive high doses of salicylate to treat certain types of advanced cancer. It reliably produces tinnitus in 95% of animals and its effect peaks after about two hours. We can then administer our test drug, again by subcutaneous injection and half an hour later, when it has taken effect start behavioural testing to measure our tinnitus marker. This testing involves playing sounds to the animal and measuring ear flick responses using a system of infrared cameras that track the ear movements in 3D space. After an hour of testing we have sufficient data to determine if the animal has tinnitus and the animal is killed by a painless overdose of pentobarbitone.

is a chronic model of tinnitus that takes about 8 weeks to develop and involves an hour long period of loud noise exposure applied to one ear using a sealed loudspeaker while the animal is deeply anaesthetised. Before and after noise exposure, while the animal is anaesthetised, we record auditory brainstem responses (ABRs) using subcutaneous needles. These needles pick up tiny brain potentials that we can use to measure hearing thresholds, before and after the noise exposure, to confirm the degree of hearing loss. Anaesthesia is induced by an intraperitoneal injection of anaesthetic mixture followed by intramuscular injections to maintain anaesthesia at a constant deep level. Animals are anaesthetised for about three hours and then recover over the following two hours. The animals all demonstrate hearing loss in one ear and are left for the next eight weeks before behavioural testing is started. About 40% of the noise exposed animals will have developed tinnitus and these can then be used to test the drugs. As it is chronic tinnitus a range of drug doses can be used on each animal followed by an hour of behavioural testing after each dose. Each dose of drug would be repeated twice in a week and a maximum of ten doses given over a period of five weeks. Some non-tinnitus animals (20% of original group) would also be given the drugs to act as controls. No animal would be tested with drugs for more than five weeks in total.

Some (60%) of the noise exposed and drug treated animals would then be prepared for terminal electrophysiology experiments. In these the animal is given a long-lasting liquid anaesthetic injected intraperitoneally followed by top ups of additional anaesthetic as required (approximately once an hour by intramuscular route). Once deep surgical anaesthesia is achieved the trachea is cannulated and the animal placed on a respiratory pump. It is then placed in a head-holder and soft tissue reflected so that holes can be drilled in the skull that will allow the placement of electrodes on the surface of the cochlea or in the cochlear nucleus. These experiments can last for up to 12 hours and at the end all animals will be killed by an overdose of pentobarbitone.



Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

High concentrations of salicylate cause tissue breakdown around the site where they are injected. Initially this is not too irritating because the salicylate is also an effective pain-killer and anti-inflammatory agent. However as the salicylate is absorbed into the blood stream and excreted by the kidneys these effects begin to wear off and will be much reduced after 8 hours. The tissue damage and associated pain still remain and will become more distressing as the analgesic effects wear off. The exact degree of pain can be difficult to assess but we know from previous work with i.p. injections that some animals can move into the severe category after one or two days. Thus we will kill all our animals within five hours of administering salicylate.

The unilateral noise loss used to induce tinnitus is a non-invasive procedure that does not seem to be associated with any appreciable pain. However the long period of anaesthesia (three hours) can be associated with complications because of the long period during which the animal is unable to adequately regulate its breathing, blood pressure or temperature. Less than 5% of animals are expected to develop problems that would cause them more than moderate distress and would require them to be killed by a schedule 1 method. The remaining animals should mostly (90%) only show mild distress, as they recover over a period of two hours following the anaesthetic, while a few (5%) may take longer to recover and or show a moderate level of distress for an hour or two during recovery.

The baseline behavioural testing does not distress the animals any more than being picked up for any other purpose. However after noise exposure some animals develop a hypersensitivity to loud sounds (hyperacusis) and this can lead to threatening or flight behaviour during the behavioural testing to determine if they have tinnitus. These animals become calm again when returned to their home cage but they have to be withdrawn from the study and are killed by a Schedule 1 method. This is expected to apply to less than 10% of the animals.

The subcutaneous injections of experimental drugs is mildly unpleasant but no animal will be given more than 10 doses of blocker drug over a period of five weeks and this will minimise any cumulative effects. At the end of the dosing regime and testing the animals will be deeply anaesthetised for electrophysiological recording and at this point they can no longer feel any pain or distress.

Anaesthesia levels are constantly monitored and there are always at least two staff present to ensure the animal never recovers consciousness.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

With the salicylate animals, all are expected to enter the moderate category but none will remain in this state for more than five hours.

With the noise exposed animals no more than 10% would be expected to show moderate symptoms of distress and the rest should only be in the mild category.



What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are trying to identify drugs that would be effective in alleviating tinnitus but before beginning clinical trials we need to obtain preliminary evidence of their efficacy using an animal model. There have been a number of studies validating the rodent behavioural model of tinnitus using human subjects - eg Fournier and Hebert (2016) The gap-startle paradigm to assess auditory temporal processing: bridging animal and human research. *Psychophysiology* 53: 759-766. Others have been able to demonstrate pre-pulse inhibition (PPI) using the small muscle potentials associated with the vestigial pinna reflex and have found similar changes to (PPI) in tinnitus patients as we see in our guinea pigs. We also know that tinnitus is a good measure of salicylate toxicity in the clinic (Samlan et al. 2008; <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2672263/>). This reassures us that our guinea pig models are closely analogous to human tinnitus. Currently there are still no drugs that have been approved for specifically treating tinnitus in either America or Europe and the clinical trials to date have still not found any drug that is broadly effective (Langguth et al., 2019; *Annual Review of Pharmacology and Toxicology* Volume 59: 291-313).

What was your strategy for searching for non-animal alternatives?

Computer modelling of the effects of the drugs on the firing rates of nerve fibres in the cochlear nerve.

Why were they not suitable?

The cause of tinnitus is still not properly understood and the mechanisms that cause its development have still not been worked out. We do not know enough about the neural mechanisms producing tinnitus in order to model the effect of altered firing rates on its development.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Effects of HCN2 blockers on behaviour following salicylate



In protocol 1 we will initially try a wide range of drug concentrations to estimate the optimal range over which a small change in drug concentration will produce a large change in the effect on behaviour. We should have one dose where there is no effect, one dose at maximum effect and then two where the effects are intermediate. Depending on the exact results the optimal range should be either between or to one side of the two intermediate doses. We will use two animals at each dose to reduce the expected variability and this means that for two drugs we will need 16 (2X4X2) animals treated with salicylate for the initial study. Once we have determined the optimal range of the curve then we will use six animals at two points on the steepest part of the dose response curve to get an accurate idea of the slope of the curve. Thus we will optimise our acquisition of dose response data as recommended in recent studies

http://people.csail.mit.edu/mrosenblum/Teaching/adaptive_designs_2011/BerryMuellerGri eve.pdf This will require a further 12 animals for each drug. This will mean that we require 40 animals specifically for protocol 1: (16 +12 +12).

Effects of HCN2 blockers on behaviour following noise exposure

The numbers required for the noise exposure model are estimated based upon the number of treatment groups and the incidence of tinnitus generated by noise exposure. In our guinea pigs about 40% of animals are expected to develop tinnitus following noise exposure. As it takes about 8 weeks for the noise induced tinnitus to develop we use 5 animals at a time and there is little risk of us using more animals than will be required to show a significant effect. Based on our previous experiments we expect that 6 animals in the tinnitus group would be sufficient and this would leave 9 in the non-tinnitus group. We estimate that we will need 15 animals for each of the two drugs to be used in the noise exposure studies. If all of these animals are noise exposed (protocol 2), then we would expect 12/30 to develop tinnitus and these would be split into two groups of 6 to test HCN2 channel blockers. We also need to confirm that the two new channel blocker drugs enter the cochlear nerve but are excluded from the brain. This will involve injections of blocker drugs, in non-tinnitus animals so that tissue can be removed at postmortem (blood, brain and nerve) to quantify the amount of blocker drug present 30 min. after administration. This will provide partition constants for each drug in the different tissues but will not require any new animals.

Effects of HCN2 blockers on neuronal spontaneous firing rate following noise exposure

Most neurophysiological measures are robust across animals and it is statistically valid to consider multiple neurons from one individual as independent, and aggregate across animals. In deciding on the number of neurons to record from we consider a response feature (spontaneous firing rate) which we predict would be higher in animals with tinnitus. If we are recording in the cochlear nucleus after the induction of tinnitus we would like to be able to detect a small change in the mean firing rate. To do this our previous calculations indicated we would need to record from about 300 neurons. In electrophysiological studies of this sort we would expect to record from at least 20 neurons per experiment. As we want to compare the tinnitus (T) and non-tinnitus (NT) animals we estimate that we will need about 15 animals in each group. In order to obtain 15 tinnitus animals for electrophysiology, we want permission to noise expose 60 animals in case only 25% (instead of the 40% expected) of the animals develop tinnitus (15 T + 45 NT = 60) but will stop noise exposures once we have obtained the required number of tinnitus animals (15 T). Thus for all three protocols we will need a total of 130 animals (40 salicylate, 30 drug effects and 60 electrophysiology = 130).



What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

By undertaking the behavioural studies first we should be able to identify which of the HCN2 blocker drugs is more effective and just use this one in the electrophysiological studies, rather than trying to compare two drugs. We have also learnt from recently published studies about how to reduce the numbers of animals required for dose response studies by minimising the numbers used to measure ineffective doses and those where the response has saturated. We have also taken advice from our local statistician about power calculations and have used the NC3Rs website to incorporate blinding into our dosing regime.

What other measures apart from good experimental design will you use to minimise numbers?

Rather than breeding our own animals, as we have done in the past, we will now only use animals, brought in from a supplier, at a specified weight. This will minimise animal wastage and reduce variability in the age of the animals that we administer drugs to. We have also undertaken extensive pilot studies using a non-specific HCN channel blocker (ivabradine) to optimise our experimental methods and routes for drug administration.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Choice of animal

Guinea pigs are placid herbivores that thrive in safe, well-maintained animal units and their sociable nature means they are very suitable for maintaining in small colonies. They have a large, easily accessible cochlea and this means that they have been a preferred model for studying the auditory system for more than 50 years. Their brains show typical mammalian characteristics that make them a good model for the human brain as they contain the same central structures and use identical neurotransmitters and modulators as humans. Additionally, there is a large amount of electrophysiological data obtained in guinea pig, and available in the literature, much of which might have to be replicated if a different animal were used (contradicting reduction). None of our protocols is rated as causing more than moderate distress and most will not produce more than mild distress.

Methods for inducing tinnitus

Tinnitus can be induced rapidly and reliably by injecting a single high dose of sodium salicylate (300 or 350 mg/kg). This causes less overall distress to the animal than noise exposure as it avoids the prolonged period of anaesthesia and subsequent recovery as well as the gradual onset of tinnitus over a number of weeks. In the past we injected salicylate i.p. and this was associated with irritation and damage to the wall of the gut that



developed over the subsequent 24 hours. We will now administer the HCN2 channel blocker two hours after the salicylate and then perform all the behavioural testing within the next two hours. This means that the animal can be sacrificed within five hours of the salicylate being administered and the pain associated with local tissue breakdown is minimised. We have made some pilot studies injecting the salicylate by the s.c. route and they did not show any signs of moderate pain. There was no vocalising during the injection or a subsequent hunched posture, immobility or piloerection of the sort we have previously observed in a few animals one day after i.p. injections. The s.c. route should be safer and cause less irritation. Salicylate injection in mice by the s.c. route has already been shown to be safer than by i.p. injection as the dose at which half the animals die (LD50) for i.p. injections is 500 mg/kg but for s.c. injections it is 550 mg/kg

<https://www.nwmissouri.edu/naturalsciences/sds/s/Sodium%20salicylate.pdf>

Salicylate is absorbed by passive diffusion and rapidly distributed in most body tissues and so the s.c. route has been shown to be effective

www.vmd.defra.gov.uk/productinformationdatabase/SPC.../SPC_261932.DOC

Identifying tinnitus in noise exposed animals requires a behavioural test and we have been successfully using a method that involves studying pre-pulse inhibition of the acoustic startle reflex. Initially we tried measuring the whole body startle which is widely used in testing small active rodents such as rats and mice. However after about the first five startle presentations the guinea pigs stopped giving reliable body movements. They still gave an involuntary ear flick and so we now use a system involving the ear flick reflex to measure changes in pre-pulse inhibition that are characteristic of tinnitus.

This is a simple procedure that allows the animal to sit quietly in a sound booth, while its ear flicks are monitored with a motion tracking system in response to startling clicks, with or without a preceding gap in the background noise that can act as a cue to indicate the imminent presentation of a startling sound. Initially we used sessions of 40 min. to monitor the ear flick responses but because of their reliability we have been able to reduce this to 20 min. sessions and still acquire valid data.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Acute recording experiments under terminal anaesthesia

All of our electrophysiology experiments will be made under deep terminal anaesthesia so that there should be no chance of the animal suffering any distress. Anaesthesia will be carefully monitored to make sure there is no chance of the animal recovering consciousness.

Guinea pigs are precocious and are born with their ears and eyes open and are fully conscious shortly after birth. There is no opportunity to use a less sentient state without the use of a general anaesthetic.

This project is designed to test the effect of drugs on tinnitus and as tinnitus is only experienced when awake the behavioural testing can only be performed on alert animals. Guinea pigs are an excellent model for human hearing because they have a similar range of hearing thresholds for low-frequency sounds and being mammals they have an auditory system and brain that are organised in a very similar way.



What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Induction of tinnitus by salicylate injection

High doses of salicylate can lead to irritation of the stomach or gut wall and we will be particularly careful to avoid this problem. We now intend to use s.c. injections to reduce the risk of damaging the stomach. Each dose of salicylate will be injected at two or more different locations in the loose skin at the back of the neck to avoid direct irritation of the gut. Animals will be carefully restrained and injections performed by trained and competent staff in accordance with LASA guidelines. The condition of the animals will be checked every half hour immediately after the injection to check for unusual sensitivity.

Recovery anaesthesia for noise exposure

The main risk of adverse effects are related to the prolonged anaesthesia required to record the ABRs and expose to loud noise in one ear. Some guinea pigs are particularly prone to the respiratory depressant effects of anaesthetics and may stop breathing even before a surgical depth of anaesthesia has been reached. By careful observation using a camera and monitor it is possible to check for a rapid decrease in the amplitude or rate of breathing just before it stops and this should allow remedial action (two drops of sublingual Dopram). Similarly the skin colour and rate of capillary perfusion can usually be checked on the fore or hind-paws either directly or with a pulse oximeter to give an indication of falling blood pressure. (This will be made easier by using albino guinea pigs rather than the pigmented ones used previously). Falls in blood pressure can be reversed by an injection of adrenaline (i.m.). Animals recovering from anaesthesia often urinate and to keep them dry and prevent any chance of local hotspots on the elements of the blanket burning them, while they are still unable to initiate movement, half a nappy will be placed under the animal. After recording the post-deafening auditory brainstem response a reversing agent such as atipamezole (1 mg) may be injected (i.m.) if appropriate and as recommended by the NVS to aid recovery. Administering a general anaesthetic may lead to mild distress due to the physical restraint or needle damage. This will be minimised by making sure staff are fully trained and monitored for competence before making injections with an assistant who will restrain the animal. After the second period of ABR recording is finished the rectal thermometer is removed and monitoring becomes more intermittent. During this time, there is the possibility that the animal will not be able to regulate temperature, or may have difficulty breathing. To protect against this, we will place the animal in a warmed recovery cage and ensure a good posture conducive to easy breathing.

Behavioural testing

Occasionally animals (< 10%) become very restless and try to bite the loudspeaker or repeatedly climb out of the cage in the sound booth during testing. This may be partly due to them experiencing increased sensitivity to sounds so that loud sounds distress them. The first time this happens testing will be stopped and the animal returned to its home cage. If it happens three times in a row the animal will be removed from the experimental group and either used in a terminal procedure or killed by an S1 method. Animals are never restrained during behavioural testing and will not experience more than a mild level of stress from being placed in the test booth. They will never be left in the booth unattended or for more than an hour in any one day. The booth has a glass window in its side so that the animal can be continuously observed.



Surgery

The respiration rate, CO₂ levels and ECG are routinely monitored to maintain a healthy physiological state. The core temperature is monitored and thermostatically maintained with a heating blanket. Some experiments have to be terminated early because of respiratory problems which we are unable to resolve. Guinea pigs are particularly prone to respiratory problems which reduce their lung capacity. Thus although varying numbers of our animals may have respiratory problems that manifest after about four hours of general anaesthesia we should still manage to acquire good data from >85% of animals. All animals will be closely monitored throughout the period of terminal anaesthesia and will not be left until death has been confirmed either by exsanguination or by monitoring heart rate and end-expired carbon dioxide levels.

Dehydration under anaesthesia will occur and we have measured that our animals lose about 1 ml of water per hour. Supplementary fluids will be administered by i.p injection of warm sterile saline as advised by the NVS.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

All non-recovery experiments involving surgery are now performed using aseptic precautions as outlined in the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery

http://www.lasa.co.uk/pdf/lasa_guiding_principles_aseptic_surgery_2010.2.pdf). This means that at least two people are present during the periods of surgery with one undertaking the sterile procedures and the other providing assistance.

These aseptic precautions include use of surgical gown, hat, gloves and sterile drapes. All instruments and drapes will have been autoclaved. A hot bead steriliser is available to re-sterilise instruments during the day if necessary but we try to avoid using it as it is no longer recommended. Procedures are in place so that the animal is never left unattended. If the fire alarm sounds (except when it is known to be a false alarm) the animal will immediately be killed by an overdose of pentobarbitone which has been placed in a syringe at the beginning of the experiment.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will rely on guidance about improved methods from the Named persons who will provide detailed oversight and assistance with all aspects of the work. They have already been working with us intensively over the last few years to help us improve our experimental methods and this will continue. We will also consult the NC3Rs website and keep informed by the relevant literature.

Explain the choice of species and the related life stages

Tinnitus does occur in children but it is more common in adults and its incidence peaks in the population decile aged from 60 - 70 years old. There is no known direct genetic link and in many people the aetiology is unknown. Thus the most appropriate animal model for drug testing is a mature mammal. Guinea pigs are a traditional and widely used model of the human auditory system. Guinea pigs have a more similar audiogram to humans than rats or mice as these small rodents have poor hearing at the low fundamental frequencies present in human speech. We have developed a behavioural test for tinnitus in guinea pigs



that is relatively quick and easy to implement and that is designed to allow us to test the effects of drugs that may alleviate the symptoms of tinnitus.



164. The health and therapeutic potential of in vitro derived oocytes using an ovine model

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Sheep	adult, embryo, pregnant, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

To test the implantation and pregnancy potential of embryos derived from oocytes that have undergone complete in vitro growth and maturation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Premature ovarian insufficiency (POI) or total sterility can occur in girls and young women as a result of genetic defects, surgery, abdominal trauma, or more commonly as a side effect of radiation or chemotherapy treatment for cancers or haematological disease. POI has adverse clinical and psychological effects on women's health. Ovarian cryopreservation can now be used to safeguard the fertility of girls and young women at high risk of sterility or POI. Primordial follicles, containing primordial oocytes, can be cryopreserved in ovarian cortex and stored long-term at -196°C. When the patient wishes



to start her family, the stored tissue is autografted back at an orthotopic or heterotopic site to restore fertility. However, for girls and women with blood-born leukaemias or cancers with a high risk of ovarian metastasis, ovarian autografting risks reseeding the cancer through the transplant. For these individuals fertility restoration can only be safely achieved in the laboratory by the complete in vitro growth and maturation (IVGM) of cryopreserved primordial oocytes into fertile metaphase II (MII) oocytes. Following in vitro fertilisation, a healthy embryo can be transferred to the patient to produce a pregnancy. The therapeutic potential of IVGM is huge but the technology is extremely challenging. Never-the-less significant advances in IVGM have recently been made to the extent that we can now derive MII oocytes from primordial follicles totally in vitro. This project will examine embryos created from these oocytes and their ability to form a pregnancy that develops normally into healthy offspring. Translation into women would have major therapeutic potential for the restoration of female fertility and the treatment of infertility. These studies will be conducted in sheep as a proven, physiologically and clinically relevant model for fertility preservation and restoration in women.

What outputs do you think you will see at the end of this project?

This research is ultimately designed to improve health outcomes. Our goal is to engage with the academic community through general meetings and academic publications, to the IVF industry, and to patient groups. Results of this research will be published in academic journals such as Nature Medicine and more specific Reproductive journals such as Human Reproduction and Fertility and Sterility. This study will impact the wider scientific community as it will provide a validated model system to study ovarian/oocyte development. With our network of scientists and clinicians we are well placed to facilitate potential impacts and prepare for the next stage of implementation. These meetings and transfer of information will effectively inform the scientific/clinical community in the area of reproductive biology and medicine. All applicants are involved in meetings that are clinically orientated and so will reach a wider audience of clinicians and embryologists and therefore potential beneficiaries.

Successful completion of this study will provide proof of concept data that will underpin the submission of an application for an HFEA research license to allow us to test the safety and efficacy of IVGM in human oocytes and embryos. Following license approval, we plan to submit a follow-up, 2-year grant application to fund safety testing of the optimised human IVGM system. Ultimately, if successful we aim to apply for NIHR efficacy and mechanism evaluation (EME) funding to test the efficacy of IVGM oocytes in a small embryo transfer trial in women.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Normal ovarian function underpins lifelong health and wellbeing in women. When it is disrupted there is a significant burden for women, their families and healthcare providers. Normal ovarian function can be compromised in girls and young women as a result of genetic defects, abdominal trauma, or more commonly as a side effect of radiation or chemotherapy treatment for cancers or blood diseases. If there is a reduction in the number of eggs formed or an increase in the number of eggs used there will be fewer eggs in the ovary and the woman will be at risk of premature ovarian insufficiency (POI) or total sterility that may have adverse clinical and psychological effects on health. Ovarian cryopreservation followed by the complete in vitro growth and maturation (IVGM) of eggs can now be proposed as a means to safeguard the fertility of girls and young women at



high risk of sterility or POI. Significant recent advances have been made in both IVGM technology and in our abilities to test the health and normality of eggs grown in the laboratory. The stage is now set to use these advances to test the therapeutic potential of in vitro derived eggs for fertility restoration. The proposed project is vital to enable selection of the best IVGM method and to ensure that the technology is both efficient and safe before it is used to treat patients.

The research project will have a direct impact on key STAKEHOLDERS that include patients who suffer from POI. The legacy of this project will also impact on academics, reproductive and developmental scientists and clinicians as well as the wider scientific community including biomedical scientists, computational biologists, and healthcare professionals. The team of applicants have established extensive communication networks in these areas that will be used to discuss and distribute the findings of the proposed studies to both the scientific and clinical communities and to the public. The unique collaborative network established for this project will impact on the MRC through realization of the potential of past, publicly funded research investments, as well as additional analyses of existing research tissue and data banks.

THE ACADEMIC COMMUNITY working on Fertility Preservation and infertility treatments shall benefit in the short term. The proposed work will also benefit young researchers and graduate students by opening up new areas of study in silico for computational biologists and will ultimately provide data which can be used by researchers in the wider scientific community to study ovarian biology and oocyte development so helping to reduce the reliance on mouse as a model system for human. Our development and use of bioinformatic tools for quality validation of transcriptome metadata will be of broad interest to other bioinformaticians, data wranglers and computational biologists who use similar approaches to analyse big data sets. The information and skill sets generated will be of value to support training provision in the analysis of RNAseq data which will enhance UK skills in the area.

ANIMAL CONSERVATION will also benefit. This project could also impact on the use of assisted reproduction techniques for endangered species. We have contacts with scientists involved in reproduction of endangered species in zoos and research institutions, both within the UK and internationally involved in reproduction of endangered species. Through these contacts our research can be disseminated to zoos and reach those interested in animal conservation management.

HEALTH CARE PROVIDERS and all those involved in Assisted Reproductive Technologies (ART) will benefit in the longer term. The results obtained could have a range of potential applications and significant commercial advantage over existing ART treatment methods. If the results of this project are positive then we will be in a position to develop methodologies for novel approaches for assisted reproduction. These could potentially a) negate the need for hormonal stimulation of the patient, b) avoid multiple surgical procedures to harvest the resultant eggs, c) implement a series of quality control measures to optimise egg selection for subsequent fertilisation and d) most importantly provide options for fertility preservation/restoration to women who have no options currently available. All these are highly desirable outcomes for improving the treatment and management of infertile women. A significant application of results obtained from this proposal would be in the area of fertility preservation. An in vitro growth system that supports the safe and complete oocyte growth and development from the earliest primordial stage to maturity could provide this patient group with sufficient numbers of viable oocytes for the restoration of their fertility in conjunction with other assisted reproduction technologies. Similar considerations may also apply to the significant number



of women with premature ovarian insufficiency, for whom a cause often cannot be found and for whom at present the only option for their infertility treatment is egg donation.

THE UK GOVERNMENT and NHS will benefit in the medium to long-term from the proposed research through: improved health and wellbeing of cancer survivors; the development of new infertility therapies; and expansion of its research base.

HEALTH POLICY MAKERS in the UK and beyond will benefit from this research in the median term through the generation of increased understanding of the ramifications of- and treatment options for- ovarian dysfunction and its contribution to lifelong health. In the long term this will positively impact on the health and wealth of the UK in terms of better health and fertility for subsequent generations, and has the potential to lessen the burden of infertility treatment costs upon the healthcare services.

THE GENERAL PUBLIC will benefit in the medium term, as we aim to disseminate our findings beyond the academic community to increase awareness of ovarian dysfunction and the need for fertility preservation for key groups of patients. By interfacing with the clinical community the project will impact directly upon health professionals through the development of new therapeutic paradigms. We will disseminate our findings beyond the academic community to increase awareness of fertility preservation strategies and by interfacing with the clinical community this knowledge will impact directly upon health professionals.

THE COMMERCIAL SECTOR will benefit in the medium term as a key driver in this application is realization of the potential of a novel therapeutic pathway as well as core and platform culture technologies and associated intellectual property. In addition to patient treatment, the core IVGM technology underpinning this application may impact on animal breeding and conservation programmes.

How will you maximise the outputs of your work?

We are regular speakers at international meetings (European Society for Human Reproduction and Embryology, World Congress in Fertility Preservation, Society for the Study of Reproduction , Society for Reproduction and Fertility, British Fertility Society etc. We have roles in organisation of meetings are invited as speakers at many basic and clinical workshops. We are members of an international expert panel who are evaluating the pathway to clinical application of in vitro derived oocytes. Project progress will be communicated directly with this international group.

We are experienced in commenting and interacting with the media (TV, Radio and newspapers) to explain research in our area of specialism and have worked with the UK Science Media Centre. We interact with our media offices to publicise research by issuing press releases for appropriate findings/publications and being available to comment on other work. All applicants are actively involved in public understanding of Science programmes, including school visits, Science festivals and informal events such as "Pint of Science". We have spoken at Progress in Educational Trust events on the impact of reproductive technologies as well as contributing to local public lecture series. We will produce web pages supporting hard copy material highlighting the research in a publicly accessible format, and will produce a video-cast of research highlight key features of the research and its potential.

REDACTED



This project will lead to the development of a data package that the business development team will use for commercial liaison.

Species and numbers of animals expected to be used

- Sheep: This will use up to 110 adult ewes and, if all embryos implanted create an offspring, up to 150 offspring

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals will have blood samples taken and have tests that are commonly done on patients when they attend the doctor. They will sometimes be given treatments by injection or pessary similar to those that are used in women during infertility treatment.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Animals undergo standard animal husbandry techniques. They will have some blood tests and measurements and be given substances in common clinical use that will cause mild transient discomfort and no lasting harm.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

The severity experienced by animals in this project is classified as moderate for the adult ewes and mild for their offspring.

What will happen to the animals at the end of the study?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?



This is the step before transfer of this technology into women. It builds of work using mammalian cells and tissues collected in vitro. This project is looking at pregnancy potential which cannot be examined in a non-animal model.

What was your strategy for searching for non-animal alternatives?

There are no non-animal alternatives as we are looking at pregnancy potential and this can only be assessed in vivo. While using cells can help refine parts of the project we need to study animals to assess the cells developmental potential in vivo.

Why were they not suitable?

We need to study animal pregnancy where multiple cellular systems interact in complex ways.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

It is important that we use just enough animals to allow us to get accurate results with minimal numbers. We look at our experience and the results from other researchers in the UK and internationally to ensure that we accurately calculate the numbers required to show changes that are clinically important. These calculations are fully displayed and reviewed during funding applications. We have planned for the minimum amount of ewes to show enough clinically significant data to facilitate transfer of the technology into women.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

The project was carefully powered by a biostatistician and the calculations were reviewed internally and externally as part of the funding application.

What other measures apart from good experimental design will you use to minimise numbers?

We use a three stage experimental model where we only progress to the next stage when the previous stage has been successful. One of the stages is a pilot phase. That means we only use animals when we have evidence that the experiments will succeed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.



Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We use clinically realistic fertility treatments and assessments that cause minimal distress and suffering without lasting harm to the animals.

We are using commercial breeders with expertise in insemination, uterine flushing and embryo transfer and all the techniques we are using for pregnancy generation and used in commercial breeding programmes. These techniques are designed to produce good quality offspring with the least pain, suffering and distress to the animals.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The ovine model has a long history of research in ovarian biology and fertility preservation. Indeed ovarian cryopreservation, which is currently in common clinical practice was developed using an ovine model. This is because sheep ovarian function and intrauterine development is very similar to that of humans.

We are studying pregnancy and the outcomes of pregnancy and as such have to use adult animals capable of becoming pregnant and being monitored through pregnancy.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We ensure that all the tissues are carefully labelled and stored at the end of the study so that ourselves and others can design new laboratory studies with already collected tissue to ensure that experiments do not need to be repeated.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We follow detailed local guidance from the LAS and national guidance from the NC3Rs and Home Office to ensure that experiments are conducted in the most refined way.

We utilise the concepts in the PREPARE guidelines: Planning Research and Experimental Procedures on Animals: Recommendations for Excellence. PREPARE covers the three broad areas which determine the quality of the preparation for animal studies: formulation, dialogue between scientists and the animal facility, and quality control of the various components in the study. We utilise the adapted PREPARE checklist.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

REDACTED has regular newsletters and meetings, at least annually, to ensure that all researchers are up to date and can implement advances appropriately. There are annual 3R competitions that facilitate sharing of best practice.

Explain the choice of species and the related life stages



We are studying the ability of the most immature eggs in the ovary to be grown in the laboratory to form eggs that are suitable for fertilisation and able to generate a pregnancy. We are looking at the health of the offspring produced. The sheep ovary is very similar to humans and the sheep has a long history in fertility research that has translated into clinical treatments for women. The size and development of sheep means that we can use the techniques and tests that are used in humans.



165. The impact of ageing and diet on connective tissue chemistry

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Mice	adult, aged, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

We aim to understand how the chemistry of connective tissue changes with age and also how diet might impact this process. We are particularly interested in the chemical changes which occur in collagen and elastin.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 unclear from the literature what the most important chemical and biological changes that occur in ageing tissue are (chemical changes are ones that occur spontaneously when two reactive molecules come into close proximity to each other, biological changes are ones driven by the biological conditions/environment, for example an enzymatically catalysed reaction which only occurs when a specific enzyme is produced). It is self-evident that changes occur with age which alter the characteristics of tissues, we can all see and feel these changes, but there is a surprising lack of clarity in the description of what chemical and biological changes are actually occurring at a molecular level.



We have shown in our own research (submitted for publication) that tendon collagen chemistry is much more dynamic than previously thought, changing in response to stretching, and that the chemistry that underlines this also changes with age. We have found that circulating glucose levels can impact the chemistry and physical properties of tendon which has led to an interest in whether diet also impacts the ageing process. Different tissues have different chemical profiles which reflect the different functions required from the tissue, for example tendon has a quite different function to bone or the wall of the aorta which is seen in the chemistry found in these tissues. In the work we plan to carry out under this licence we wish to continue to study connective tissues to understand how the changes in the chemical properties seen as the result of ageing and diet impact the normal functioning of the tissue.

Understanding this may lead to improvements in treating diseases such as diabetes and cystic fibrosis, as well as potentially providing a rationale for strategies for healthy ageing or tissue repair.

What outputs do you think you will see at the end of this project?

We will publish the results of our animal work in respected peer reviewed journals.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

A better understanding of the processes involved in ageing could ultimately help in both treatment and prophylaxis of diseases associated with normal ageing. It is expected that this project will lead to the development of methods to study ageing tissues and identify the important changes which occur as we age. Ultimately we aim to transfer the methods developed here to study the ageing of tissues in humans.

It is likely that changes in connective tissue chemistry impact many disease processes. For example, changes in crosslinking and glycation in the collagen of arterial walls are expected to lead to increased stiffening leading to increased blood pressure, an increased incidence of aneurisms and arterial valve stenosis. With respect to ageing, the stiffening of joints and tendons would also be expected as a consequence of increased collagen crosslinking and glycation. The treatment of aspects of diseases not necessarily linked to ageing, such as lung damage in cystic fibrosis and poor wound healing and tendon ruptures in diabetes may also benefit from this research.

The tissues generated in this project will be stored and made available to other researchers interested in the ageing of connective tissue, providing a valuable and unique resource of isotopically labelled and chemically profiled tissues of different ages. This will be done through personal contact with other researchers in this field of research. It is reasonable to expect that other researchers studying normal ageing processes would find this resource valuable. We also try and time our experiments so that other researchers within the organisation can make use of fresh tissues when we kill aged animals, such as lymph nodes, bone marrow etc.

How will you maximise the outputs of your work?

We collaborate with other researchers on the ageing of connective tissue and give presentations on our latest results when appropriate. This is the best way to disseminate information on approaches that do not work.



Species and numbers of animals expected to be used

- Mice: 650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The mice will be kept for up to an age of 130 weeks and fed balanced diets. In some cases mice will be put under a light general anaesthesia so that ultrasound measurements can be made to determine the stiffness of the cardiovascular system. These cardiovascular measurements may be carried out up to 4 times with a minimum period of 4 weeks between each measurement.

Mice will be kept under standard animal house conditions up to an age of 130 weeks. db/db mice and Nrf2^{-/-} mice will be purchased at various ages and kept for the minimum period of time to allow a single cardiovascular ultrasound measurement to be obtained from each animal. This will be no longer than 1 month.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

There are no specific impacts or adverse effects expected on wild type mice ageing healthily during this project. The majority of the animals used on this project will be wild type. Animals that are developing irreversible ill health conditions will be killed before they exceed a mild threshold because we are interested in healthy ageing and not the ageing of diseased or unhealthy animals.

We have kept mice routinely to 98 weeks with an upper limit of 104 weeks and found that these animals have been remarkably healthy and could have been kept longer without causing harm.

Two standard genetically altered strains from commercial sources will be used in addition to the wild type animals. One strain (db/db) is diabetic and the other (Nrf2^{-/-}) has a poorer immune system linked to a mutation that causes reduced levels of glutathione. These will be monitored closely and kept for the minimum time required to make a single cardiovascular ultrasound measurement, and no longer than 1 month. These animals will only be fed a normal standard diet typically used in the animal unit.

The animal house staff are trained and experienced in the recognition of signs of ill health found in ageing animals.

Animals are inspected during routine cage cleaning with in hand inspection weekly for mice over 52 weeks old.



The staff will be looking particularly for eye abnormalities, malocclusion, over grooming, ulcerative dermatitis, abscesses, rectal prolapse, weight loss (a potential sign of other underlying problems), neurogenic symptoms (fits / seizures / spinning / head tilt), abdominal swelling. These are conditions that could develop as a result of old age and diseases expected to be found in old age.

Any animals showing signs of illness which, in the opinion of the NVS, cannot be ameliorated with minor interventions will be killed using a Schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

The mice are not expected to exceed a severity of Mild.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 ageing of tissues is a complex process involving a multitude of cell types and factors. For example, a blood vessel like the aorta is a complex highly organised structure of cells, collagen, elastin and other connective tissue components. The blood vessel wall is not believed to have a fixed composition at a molecular level, changing with age and in response to factors such as blood pressure. Similarly, the structure of other tissues such as tendons and bone respond to stresses and age related factors which it is not yet possible to reproduce in culture.

What was your strategy for searching for non-animal alternatives?

There are no alternatives that I am aware of that could be used to study changes to the chemistry of connective tissue during ageing.

Why were they not suitable?

There is no alternative model that has the complexity of the interacting biological processes found in animals during ageing.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise



numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is based on the experience of running a project over the last 5 years and typical animal usage from our records. We involve a professional biostatistician in the design of experiments to minimise the use of animals wherever possible. Many of the animals used are to provide fresh aged tissues for study after they have been killed.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We typically start experiments with a small number (5 to 10) animals in each group, collect the required data and then collaborate with a statistician to decide if we need to increase the number of animals used. We then collect data from additional animals and add this to the original data set, if appropriate.

What other measures apart from good experimental design will you use to minimise numbers?

Where possible we keep the carcasses of the animals that we use so that we can sample tissue from different organs. This helps to minimise animal usage and allows us to build up a picture of changes in the connective tissue across the whole body of individual animals. For example, this potentially will allow us to correlate changes seen in skin with changes in the aorta, tendon or bone.

Where old and young animals are killed by other researches for tissues and the carcasses are unwanted, we often take these and keep the remaining tissues that could be of use in our 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The mice are allowed to age normally under this project and so they should not experience anything that a normal healthy mouse would not.

Where we require cardiovascular ultrasound measurements the mice will be put under general anaesthesia.

We have limited the time that commercially available genetically modified animals can be kept to minimise the potential for suffering or distress. The genetically modified strains required are extensively used by others and the phenotypes characterised.



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We are trying to understand why connective tissue ages and relate these changes to those seen in humans. The animals chosen are required to have a biology that is as close to human biology as possible. Mice fit this requirement.

The extracellular matrix found in non-mammalian species is chemically different to that of mammals which makes them unsuitable as an alternative. The physiological environment within the extracellular matrix, organ structure and diet also effect the chemistry of the matrix. These are too dissimilar in non- mammalian species.

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

The mice should not experience any harm.

In this project we use two strains of commercially available genetically modified animals. One strain is diabetic (db/db) and the other has reduced glutathione levels (Nrf2-/-) with increased risk of infection. These two strains of mice will be kept for less than 1 month to enable the required measurements to be made and minimise the potential for harm to the animals.

Neither the db/db mice nor the Nrf2-/- mice will be fed altered diets. The db/db mice will not be fed diets containing raised levels of sugars.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The NC3Rs website contains a wealth of information on best practices, experimental design and animal welfare.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Through reading of literature and listening to presentations on advances in the 3Rs.

Explain the choice of species and the related life stages

We are studying the chemical changes which occur in connective tissues (primarily to collagen and elastin) with age. As such we require mice of all ages. Ageing mice are extensively used as a model for human ageing because they show the same attributes of ageing as seen in humans, but over a shorter time frame.

We have shown previously in tissue from db/db mice that higher levels of glycation are seen without increases in other crosslinking. This is required to understand the mechanism and impact of collagen glycation on function without the complication of other increases in crosslinking typically associated with changes in tissue function.

Nrf2-/- mice are required to test a hypothesis that glutathione levels play a role in crosslink reduction in elastin found within the extracellular matrix.



166. The importance of gut microbes in health and disease

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.

Key words

No answer provided

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

This project aims to understand how the populations of microorganisms resident in the gut (the microbiota), contribute to health and disease, and to use the knowledge gained to develop the means of altering the microbiota to improve or restore health and to treat diseases affecting the gut and other organs, such as the brain.

The key objectives are:

To determine how gut microbes and their products communicate with the cells in our body to influence their behaviour and function.

To determine the most effective means of changing gut microbe populations to promote or restore gut health and treat disorders affecting the gut and other organ systems.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?



The expected benefit of this programme of work is to provide an increased understanding the role that gut microbes play in promoting healthy ageing that will lead to new interventions to promote or boost gut health in at risk (elderly) individuals.

One such anticipated intervention and benefit from our research is the development of new vaccines. There are currently only a handful of licensed vaccines able to protect the major sites of pathogen entry to the body - the gut and lungs. New vaccines are desperately needed in order to prevent infection by respiratory and gut transmitted pathogens that collectively account for more than 8 million deaths annually worldwide. In particular, there is an urgent need to develop new prevention and treatment strategies for viruses such as influenza and coronavirus that can cause pandemics (i.e. COVID-19). The young and elderly are at particular high risk from these infections due to their weakened immune systems that have a major impact on the NHS and social care expenditure. By genetically engineering a major bacterium that resides in the human gut to express virus vaccine antigens in its' microvesicles (outer membrane vesicles; OMV) that are shed from its surface we have generated OMV containing vaccine antigens and therapeutic proteins for oral and nasal administration. This project will enable us to further optimise and refine OMV formulations and test their effectiveness in providing increased resistance to infection and disease prior to their use in humans and in veterinary medicine.

Another anticipated impact of our programme of work relates to manipulating gut microbes to prevent or treat age-associated decline in immune function and mental health (cognitive function). Events taking place in the gut play a central role in the ageing process and in the functional decline and senescence of various organ systems including the immune and cardiovascular system, and the brain. This makes gut microbes a realistic target for interventions to positively impact on the ageing process. As part of this project therefore, we will further refine and develop microbiota replacement therapy (faecal microbiota transplantation; FMT) by determining its mechanism(s) of action and optimising its effectiveness. We will also investigate other approaches to manipulating gut microbes including the use of single or defined mixtures of microbes or microbial products such as OMV. We anticipate that this new knowledge and data will during the lifetime of the project be used to develop evidence based protocols that can be used in clinical trials to assess the efficacy of FMT or other forms of microbe-based therapies to overcome immunosenescence and boost immunity in elderly individuals.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Mouse, approximately 10,500 over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The germfree or GA strains we will use are not expected to develop any adverse clinical or psychological signs. However, in the they show any deviation from normal health or



wellbeing will be immediately killed by schedule 1. Aged animals (>20 months) are expected to show signs of frailty and immunosenescence that is a hallmark feature of aged mammals. Frailty is assessed and scored by monitoring an individual animals weight and its movement over a fixed period of time. Those that do not remain in good health and display generally accepted end of life signs (e.g. slow or laboured respiration, hunched body position with matted fur, failure to eat and drink determined by food hopper weights and degree of dehydration or, poor body condition score) will be killed by schedule 1. Clinical presentations for aged mice include rectal prolapse, alopecia and dermatitis, ocular lesions and palpable masses although the presentation and severity of these signs can be varied making it difficult to predict the expected incidence. However, any animals displaying any of these signs may after consultation with the NVO be killed by schedule 1.

We will administer substances that include harmful and harmless microorganisms and/or their products or antigens, chemicals, and drugs such as antibiotics and steroids to induce inflammation and colitis that replicates key features of intestinal inflammation and inflammatory bowel disease in patients. These substances will result in no lasting harm in the majority of animals (>90%) using the minimum dose consistent with the scientific objectives. Doses will be no more than the maximum tolerated dose (MTD - defined as that associated with weight loss of no more than 20% of the animal's initial bodyweight). All mice receiving these substances will be inspected and weighed daily. Mice will be promptly and humanely killed if they are approaching 20% weight loss and/or display signs of pain, distress or of significant ill health such as abnormal posture/positioning, abnormal breathing, decreased food/water consumption, dehydration, muscle rigidity, twitching/trembling or pilo-erection.

The treatment regimens used to prevent and/or treat inflammatory disorders affecting the GIT and its microbiota, the immune system and the CNS are expected to improve overall physical and/or mental health and we do not expect them to exacerbate or worsen any pre-existing symptoms or illness. In the unlikely event they do, animals will be promptly killed by schedule 1.

Behavioural tests are used to assess motor, cognition and motivational skills and have been chosen over more aversive tests as they have been extensively employed to assess the impact of genetic modification on brain function without requiring the traditional motivational constraints of shock or food deprivation. None of the tests to be used are expected to cause any pain, suffering, distress or lasting harm in well habituated animals. Aged and frail animals may however exhibit excessive signs of distress during these tests in which case they will be immediately withdrawn. If the signs persist they will be killed by schedule 1.

Withdrawing food and/or water for short periods of time and up to a maximum of 12 h for food withdrawal and 4 h for the withdrawal of water should result in no discernible adverse effects. Since rodents consume most of their daily food intake during the dark phase we would fast them during the day to minimise distress. Any animal showing any deviation from normal health or wellbeing will be immediately killed by schedule 1. The administration of modified diets is designed to test the ability of the animals to resist obesogenic diets and some will therefore gain excess weight over the study period. However, a 12 week study period is sufficient to detect an effect of the diet on metabolism and the development of metabolic syndrome, but not long enough for excessive weight gain to cause distress or significant ill health.

Insulin and glucose resistance testing is a well-established method for assessing metabolic status and diagnosing metabolic syndrome. It is not expected that any mice will



experience any adverse effects as a result of insulin or glucose administration. However, blood glucose levels will be monitored (at least every 30 minutes) for 2 hours after administration, by which point blood glucose should return to baseline levels. If levels do not return to baseline within 2 hours or if at any time animals display significant clinical or psychological signs of ill health they will be immediately killed by schedule 1.

All animals anaesthetised for imaging purposes are expected to make a full recovery. The interval of repeated general anaesthesia (a minimum of 24 hours) enables animals to make a full recovery as determined by exhibiting normal behaviour, eating and drinking normally and socialising with cage-mates. Any animals failing to fully recover or that develop signs of significant ill-health such as breathing difficulties, inappetence, hunching, crouching or pilo-erection, will be promptly killed.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

The reasons why this project cannot be undertaken without using protected animals are:

Computer modelling and systems biology is not sophisticated enough currently to account for the complex multi-organ interactions or the dynamic activity of multiple cell types within an individual tissue the function of which is determined by their spatial organisation within complex tissue architecture.

Laboratory-based assays cannot faithfully replicate or model the complexity of tissues and cell types present in the GI-tract that is integral to its normal function.

Laboratory-based assays cannot faithfully replicate or model the interactions involving gut microbes and the myriad of cell types that reside in gut.

Many different types of cells in the gut cannot be kept alive outside of the body and cannot be kept alive in the laboratory.

A significant proportion of gut microbes cannot be kept alive outside the body and in the laboratory.

The gut contains cells comprising the immune system, the nervous system, the endocrine system and multiple types of barrier epithelial cells each of which has their own specific requirements and conditions for growth making it very difficult to study them combined in laboratory-based cell culture systems.

The intervention studies proposed in this project cannot be performed in humans due to ethical constraints.

As part of this project we will evaluate the use of newly emerging technologies that might overcome or minimise the factors identified above that would allow us to reduce our animal use. This will include the use of intestinal organoids, organ (gut/brain)-on-a-chip technology, and advanced fermentations systems for culturing intact complex microbial communities.

Reduction



Explain how you will assure the use of minimum numbers of animals.

When designing experiments we rely on 30years past experience, literature searches, consulting the institute statistician and statistical analyses to ensure the minimum number of mice per group that will be informative are used. The exact numbers of animals required will for example vary with the particular experimental design, the estimate of variation in response, and other factors. For qualitative experiments, the amount of material required will be the minimum necessary to provide an adequate description.

To reduce the number of breeding pairs, genetically altered (GA)-mice expressing the mutant gene will be kept wherever possible, provided they remain healthy and able to breed. Breeding schemes will be designed to use the minimum number of breeders to obtain sufficient numbers of age and sexed matched animals of each of the required genotype (wild type, heterozygote, homozygote)

To maximize the information from a single animal and to minimize suffering, we will aim to collect samples post mortem, we will share tissues with other appropriate scientific colleagues for use in their work.

We will as part of this project evaluate the use of newly emerging technologies that would allow us to reduce our animal use. This will include the use of intestinal organoids, organ (gut/brain)-on-a-chip technology, and advanced fermentations systems for culturing intact complex microbial communities.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The mouse is an appropriate and extensively validated model for gut biology and disease and is the species in which reliable genetic engineering technology and germfree experiments is best established. Also, mice are our model species of choice based upon our 30years personal experience of undertaking these experiments. They are also the standard species for producing genetically altered- animals and are the de facto standard for this work globally.

Prior to embarking on animal experiments we will collect as much evidence as possible to determine whether a candidate gene or microorganism is likely to effect gut health and immunity. This will involve collaborating with colleagues that have expertise in gut microorganisms and intestinal organ, and isolated epithelial cell culture. While these techniques hold considerable promise they cannot reproduce the interactions of more than two cell types.

Ageing is major focus of this project and requires the use of aged animals (>20 months). Aged mice will be closely monitored for signs of ill health and any displaying generally accepted end of life signs including non-responsiveness to being touched, cold body temperature to the touch, slow or laboured respiration, hunched body position with matted fur, failure to eat and drink (determined by food hopper weights and degree of dehydration) or, poor body condition score or approaching 20% loss of body weight relative to baseline will immediately be killed by a schedule 1.

Wherever necessary local and general anaesthesia will be used to minimise animal suffering. In all long-term experiments, animals will be promptly killed if they are



approaching 20% weight loss and/or if they show any signs of ill health during experiments such as hunching, lack of group behaviour or breathing difficulties.

Microorganisms will be administered at doses known to induce an appropriate inflammatory response. Where the outcome of infection is unknown, dosage will be carefully titrated using small-scale trial studies to minimise any adverse unknown effect.



167. The influence of metabolic disturbances on platelet function, thrombosis and vascular 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
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

Blood platelets play critical roles in the prevention of blood loss, termed haemostasis, but also participate in pathological thrombosis, cancer and cardiometabolic diseases. This project aims to dissect the molecular mechanisms unpinning platelet driven disease processes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Blood platelets are cells that become sticky and clump together to form blood clots to stop us from bleeding after injury. However, in some people platelets also form blood clots inside intact blood vessels leading to heart attacks and strokes. The formation of these



blood clots, called thrombi, account for large numbers of deaths in the UK. We know that people at risk of these diseases often have increased levels of fat and sugar in their blood, and that raised fat and sugar concentrations may cause platelets to form clots more readily in the blood stream. However, we do not know how these fats and sugars cause excessive blood clotting. If we can identify the proteins on platelets that respond to fats and sugars it will help doctors understand the causes of blood clots and to develop new medicines to prevent thrombotic disease. The only way we can really examine the importance of these proteins is to increase the amounts of fat or sugar in blood of mice and then determine how blocking or deleting them affects blood clotting. Therefore the work we propose is critically important to help doctors and the pharmaceutical industry to develop new strategies in preventing a major cause of death in the UK and worldwide.

What outputs do you think you will see at the end of this project?

Our key endeavours are academic excellence, unravelling the molecular mechanisms that drive platelet hyperactivity, which could be used to improve the lives of patients at risk of thrombotic diseases. For example, in the lifetime of our previous licence we published two high profile papers describing novel mechanisms of platelet activity and how in principle they could be targeted therapeutically for clinical benefit. Our primary outputs will in the form of academic publications describing these mechanisms. We hope that these publications act as platforms for further studies by our group, and others, to translate our findings to patients at risk of thrombotic disease.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Pathological thrombosis is a major component of the pathology that underlies cardiovascular diseases (CVD) and other chronic diseases. In the UK, CVD accounts for 28% of annual deaths is the greatest cause of mortality, killing approximately 161 000 people in 2012 (www.heartstats.org - British Heart Foundation). Furthermore, these numbers do not take into account the thrombosis related deaths associated with diseases such as cancer and diabetes. Thus, thrombotic disease inflicts a significant health and financial burden on the UK.

The beneficiaries from these output will be varied and include the academic, clinical and pharma sectors. The outcomes we generate from our programme of work will provide new knowledge of platelet driven disease processes providing further opportunities to drive academic excellence. A second element of this will be the generation of new animal models, for example, the generation of platelet specific genetically modified mice that may be applicable to other areas of research including diabetes, cancer and immunity/infection. As is standard with good academic practice all models developed in our laboratory would be shared with the wider scientific community, both directly within our institute and wider afield. Should our work lead to refinements in laboratory practice for example as improved surgical, imaging or experimental procedures these would be made available for other researchers to utilise. Our work programme is focused on a key unmet clinical need. Further exploration of the mechanisms of platelet hyperactivity would allow clinical colleagues insight into the potential causes and management of disease. There are also potential advantages for the pharma industry, who remain highly interested in developing agents to control unwanted platelet activation.

Therefore, the project will continue to help us identify the specific roles of the proteins that regulate or inhibit platelet function and allow us to evaluate their potential as targets for the



development of new antiplatelet drugs that could reduce/prevent heart attacks and strokes. While we have no guarantee that the proteins studied will in the future become useful therapeutic targets, it will add to the overall of knowledge of this area of research and help other researchers, both in the UK and internationally, who are focussed on solving problems associated with thrombosis.

How will you maximise the outputs of your work?

We will publicise our findings to the wider scientific community, industry and the public through annual conference presentations, publications and through other media.

Our work is presented on a regular basis within our institute, through research seminars, and often disseminated through academic conferences. These are used to discuss the work in progress and provides a forum with which we can discuss experimental issues (including animal welfare and phenotypes) informally with other experts in order to refine techniques and to help develop best practice. The publication of our work is another major form of dissemination, where we report the findings in a more formal manner. In our recent work we used a similar approach to that outlined in this application to identify a new mechanism of thrombosis.

We aim to publish at least two high impact publications, which build on our previous work. Upon publishing our research results in scientific journals, we will prepare press releases in collaboration with the press offices of the REDACTED. This transparency of approach to sharing of data will maximise our outputs, ensure that our research activities are complementary rather than competitive and that the field moves forward as quickly as possible.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The mice in this part of the project will undergo an altered feeding regimes for up to 16 weeks and then blood harvested under anaesthesia. The procedure will be performed once per animal followed by termination under an approved schedule 1 method.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

There are three areas where adverse effects maybe anticipated.

Dietary manipulation - on rare occasions some mice find high calorie diets unpalatable and begin to lose weight. Mice will be monitored daily for their general well-being and weighted on a weekly basis.



Should an animal show signs of distress they will be removed from the study returned to a normal diet. If the distress continues the animal will be culled by a schedule 1 procedure.

Administration of metabolites/drugs - There could also be potential impacts of administration of substances designed to influence the recruitment of circulating platelets and/or thrombosis. Substances will only be administered where dosing and potential toxicity data are available in the mouse or similar species. Such substances, such as established drugs or metabolites, are therefore not expected to produce adverse side effects at the concentrations administered. It is anticipated that the drugs used in the proposed experiment will be given intravenously on three occasions prior to harvesting of blood. If adverse effects are suspected the animals will be withdrawn from the study. If the adverse reactions persist the animals will be killed by a Schedule 1 or other method stated in the protocol immediately. As data is collected we will review the protocol to ensure that the severity limits are appropriate.

Recovery during anaesthesia - The severity of this procedure will be controlled through use and careful monitoring of general anaesthesia throughout. There a possibility the animal recovers prematurely from anaesthesia, although this would be a relatively rare occurrence. To prevent premature recovery from anaesthesia there will be continuous monitoring of the depth of anaesthesia by testing the limb flexion/withdrawal reflex and/or the corneal reflex, which will be supported continual monitoring of heart rate and body temperature. Should there be suspicion that the animal is recovering prematurely additional anaesthetic will be administered by inhalation. However, it is noteworthy that this has not occurred during the procedures performed in the last five years and therefore we do not anticipate this to be a common occurrence.

In our experience all of the possibilities highlighted above are rare.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

The severity level for this protocol for all mice is mild and performed under terminal anaesthesia.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 blood platelets is dictated by three limiting factors.

There is no reliable in vitro tissue culture models for the generation of platelets from primary megakaryocytes or cell lines hence the only source of platelets is from an animal model.



Platelets are not amenable to genetic manipulation since they lack a nucleus. Therefore, transfection studies used by many other researchers as an alternative to animal experiments that work with nucleate cell systems are not possible.

Therefore researchers commonly use genetically altered mice to identify and delineate the pathways involved in platelet activation. This laboratory is committed to perform work with animals only when the potential biomedical advances warrant this. We are interested in developing new approaches that would reduce the use or generation of genetically altered mice to study platelet function, and we are monitoring closely studies in other laboratories that have begun to examine ways to produce genetically modified platelets (using siRNA technology) *in vitro* from bone marrow culture. Should this technology become available will look to adapting it for our own studies.

What was your strategy for searching for non-animal alternatives?

None

Why were they not suitable?

They lack recognisable platelets.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In the types of studies, we perform the anticipated response is usually 'all or nothing', that is the genetically modified mice fail to respond to stimuli when compared to the wild type. Therefore the numbers for *in vitro* studies of our programme are calculated based on the numbers of samples we can prepare per mouse. For example, the blood from each animal provides approximately 1 ml of washed platelets which is sufficient to prepare for four samples. With this knowledge we can extrapolate to the appropriate number of mice required by determining how many samples are required for each series of experiments within our project. Under these controlled conditions we aim to use approximately 6 mice per strain per assay (and 6 control animals), since platelet responses are generally very reproducible. In general we use between 10 and 15 assays during our functional evaluation.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Our experimental design is driven by studies performed in human platelets, which act as a guide to the potential importance of key proteins in the function of platelets. Careful analysis of the data emerging from these experiments will determine the need for experiments in animals. It is noteworthy that the experiments performed under this licence are *discovery science* and so very little information is available to guide our calculation of numbers. However, given the "all or nothing responses" expected, allows us to ascertain



with relatively low numbers of mice whether extensive experiments using multiple in depth assays are required.

What other measures apart from good experimental design will you use to minimise numbers?

We have a number of strategies for optimising animal numbers.

The key step is the generation of pilot data from *in vitro* functional assays, lack of phenotype in these assays (usually between 10 and 15 assays) prevents mice being used in experiments that require dietary manipulation and reduces the breeding of mice.

All tissues from mice sacrificed under any of the protocols are available for other researchers within our institute.

All genotyping is performed by a commercial supplier to allow for industry standardised protocols. Correct genotyping is critical to implementing a targeted breeding strategy that allows for good colony management.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Currently the mouse haemostatic system is considered a strong model for that found in humans and has the advantage of genetic manipulation. Therefore the project will use mice for the proposed studies.

The project uses two key steps to determine how specific proteins affect platelet function, all of which have been designed to avoid suffering and distress in the mice

Dietary interventions are mild and therefore do not induce an extreme metabolic phenotype.

Harvesting of blood is performed under anaesthesia and without continued use, which again minimise suffering to the animals

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Non-sentient animals cannot be used for our studies since they lack recognisable platelets.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?



The major refinement to our protocols is in the area of blood harvesting from mice. We have now moved away from the use of cardiac puncture and take blood from the inferior vena cava. The success rate of harvesting blood using this method is significantly higher and more reproducible, thereby reducing the numbers of mice required for experiments. Furthermore since this procedure is performed under terminal anaesthesia it minimises suffering in the mice.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Major guidance through the project is taken from the NC3Rs, whose website (hubs and microsites) provides a vast array of resources on the general principles underlying the experiments highlighted in this project; this includes anaesthesia, breeding strategy and numbers, and experimental design.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The applicant's laboratory is conversant with the 3Rs and in continually examining ways that the principles are implemented. To ensure this

Group members are encouraged to check the NC3Rs resources page

We are kept updated by the NIO on a regular basis regarding changes in best practice, courses and additional training.

We are aware of the scientific literature in the haemostasis field, where refinements to these types of procedures are reported.

Where changes/advances in practice have been reported we aim to up-skill either through training courses or visiting laboratories to receive guidance.

Explain the choice of species and the related life stages

Mice are the smallest mammal with a haemostatic system similar to that of humans and therefore is an excellent model for evaluating thrombosis and haemostasis in vivo. The murine model has the added advantage of being amenable to genetic manipulation allowing the functional characterisation of specific proteins.





168. The long-term effects of developmental hypoxia on cardiac function

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

Heart disease, Prenatal hypoxia, Antioxidants

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The overall aim of the project is to investigate the long-term effects of insufficient oxygen supply to an unborn baby. Our specific aims are;

To understand the long-term effects of insufficient oxygen on the structure and function of the unborn heart

To study the suitability of a class of drugs called "antioxidants" to protect the unborn heart from a lack of oxygen and prevent the development of cardiovascular disease later in life.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

The main benefit of the study is the advancement of current understanding of the underlying mechanisms that lead to heart disease in babies that received an insufficient oxygen supply in the womb. We hope to identify novel targets for drug intervention to protect people from developing cardiovascular diseases later in life. All of the findings will be published in peer-reviewed leading scientific and clinical journals as appropriate to ensure wide dissemination of the research findings. The information is of direct benefit to



physiologists and clinical cardiologists and will provide key information enabling better management of cardiovascular disease.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Approximately 1110 Wildtype rats, and 30000 zebrafish over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Up to 70 pregnant rats will be placed in a chamber for up to 16 days where oxygen levels will be set between 9-21%. This level of oxygen will cause tissue hypoxia in the pregnant rats (known as prenatal hypoxia) and unborn offspring (known as fetal hypoxia). Most of the pregnant rats subjected to prenatal hypoxia will experience procedures which fall under the mild severity banding, including; 1) transient discomfort from blood sampling needles, 2) transient throat irritation (from intubation) and 3) a transient reduction in water intake. A lesser proportion of pregnant rats will experience symptoms which fall under the category of moderate severity banding, including; 1) a transient reduced activity, 2) a transient reduction in food intake and body weight, and 3) a transient feeling of being unwell from maternal pre- eclampsia-like symptoms. In addition to maternal adverse effects, most of the pups (up to 550 rats) will experience procedures which fall under the category of moderate severity banding, including; 1) a transient or permanent intrauterine growth restriction (IUGR) which causes a reduction in body weight, and 2) a permanent increase in disease susceptibility. In very rare cases, pups will experience permanent physiological and morphological birth defects. All of the zebrafish in this study (30,000 fish) will experience a transient or permanent reduction in body weight caused by hypoxia. At the end of experimentation, all of the pregnant rats, some of the rat offspring (950 rats) and all of the zebrafish will be humanely killed. The rest of the rat offspring (150 rats) will be delivered to a collaborating establishment for a separate, parallel set of experiments; at the end of these experiments, rats will be humanely killed.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

We need to use animals because our study is assessing the long-term effects of prenatal hypoxia (up to a year) which cannot be studied in cell lines, nor can they be suitably modelled using computer simulations. It is not possible to use human volunteers because human tissue is of limited availability. Lastly, we cannot use non-protected animal alternatives because we wish our findings to be clinically relevant to human diseases of the heart, and the use of other less sentient species, such as reptiles, fish and amphibians, is usually not appropriate for the main study animal as their hearts differ significantly from



mammalian hearts. Nevertheless, we have utilised the zebrafish which are naturally tolerant to hypoxia to understand adaptive responses.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Experimental design has been discussed with, and approved by, our statistical advisor. In order to minimise the number of animals required, sample size has been estimated for each investigation based on existing published data and the use of power analysis. These estimates will be updated and recalculated throughout the project as we generate new data.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Experiments concerning rats:

We have chosen the rat as our main experimental species for several important reasons:

Rats reach reproductive maturity quickly and they have a short lifespan (2 years) which allows the long-term effects of prenatal hypoxia to be studied within a reasonable timeframe

The rat model of prenatal hypoxia is well-established; there is a wealth of information to base our investigations on.

My collaborator has already determined that antioxidant therapy reverses the negative effects of prenatal hypoxia which gives us an opportunity to develop drug treatments to protect the heart.

Steps to minimise welfare costs to animals:

It is not possible to house pregnant rats in groups because we need to monitor food and water intake during the study; but once pups have been weaned, rats will be housed in stable, compatible groups.

The following parameters will be measured during the protocol to ensure animals remain within the outlined severity limits: Body weight, body condition scoring (BCS), food and water intake and cardiovascular status.

Control animals not subjected to any procedures will be used as a benchmark for normal changes in these parameters.

For moderate levels of prenatal hypoxia (below 13% oxygen), rats will first be put into the chamber at normal oxygen levels (21%) for 24 hours, and then oxygen will be reduced slowly (over another 24-hour period) to avoid shock.

The following rules will be applied to rats transported to collaborator establishments;



Prior to transport, all rats will undergo a health check by local NVS and at least 2 of the rats will be screened for viruses and pathogens.

Rats will be transported according to the NC3R's best practice for animal transport, and in line with UK legislation according to the The Welfare of Animals (Transport) (England) Order 2006. We will use a reputable courier who is regularly used by the establishment.

Upon arrival at the collaborator's establishment, another health check will be made by the collaborators NVS

Experiments concerning fish:

Similar to rats, fish have a short lifespan which make the suitable for longitudinal studies, but they also routinely experience developmental hypoxia in the wild, and they are known to mount adaptive responses to hypoxia. Studying these species will allow us to identify adaptive mechanisms which could be manipulated in mammalian hearts to provide protection against hypoxia.

Steps to minimise welfare costs to animals:

Zebrafish are extremely hypoxia tolerant, so we do not expect any adverse effects from hypoxia exposure. Nevertheless, we will regularly monitor the animals for signs of illness or disease.



169. The mechanics and energetics of locomotion in birds

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Galliformes (e.g. chicken, guineafowl)	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 is the aim of this project?

The overall aim of the project is to determine how muscles are used during locomotion and how this determines the overall amount of energy used (or effort) during exercise. These measurements will underpin efforts to develop methodologies for estimating energy expenditure in freely living birds.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Improving understanding of the energy used during locomotor is of broad scientific relevance and will have impact on a range of disciplines, from ecology to engineering.

Recent advances in engineering means that it is becoming possible to build legged-terrestrial robots and flapping air vehicles that operate on a similar scale to birds. However, their manoeuvrability, control and energy use are still far below that achieved in nature. Understanding bird locomotory systems will provide inspiration towards the development of bio-inspired robotics and more generally the design and control of micromechanical systems.



Such knowledge will also help in the development and refinement of computer models of animal movement. Ultimately, the research will contribute to the replacement, reduction and refinement of animals in testing since the development accurate computational models will allow some animal experiments to be replaced and in other cases reduce the numbers of animals required, as model simulations may allow research involving animals to be better designed.

There increasing interest in the changes in the distribution of organisms in response to climate change. Energy expenditure during movement is an important factor that could influence their migratory paths, distribution and ultimately their survival of birds. An improved ability to study energetics in the field will provide a useful tool to help explain current changes in population and species distribution and in predicting which species are likely to be adversely or favourably affected by future changes in climate.

What outputs do you think you will see at the end of this project?

Research publications targetted at the leading journals in the field.

Conference presentations at leading national and international conferences in a variety of fields to maximize impact.

Practical tools that will allow researchers to better estimate organismal energetics in the field.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Academic beneficiaries (Years 1-5): the research will be of interest to a range of biological fields, including physiology, biomechanics, ecology, robotics, computational biology. For example, ecologists studying the links between animal morphology and ecology and those interested in using practical tools for making informed assessments of bird energy use in the field.

General public (Years 1-5): animal locomotion is a topic that consistently arouses public interest. The research will inspire young audiences to take an interest in science and will have a positive impact by informing the general public about technological advances in animal science and the applications of biological research.

3Rs (Year 5): Developing accurate computational models of locomotion may allow some animal experiments to be replaced and in other cases refined or reduced as model simulations may allow research efforts involving animal research to be better designed.

Conservation NGOs (Year 5): An improved ability to study energetics of locomotion in the field will provide a useful tool, alongside ecological and developmental factors, to help explain current changes in population and species distribution and in predicting which species are likely to be adversely or favourably affected by future changes in climate. The rapid increase in the number of migratory species being studied has revealed the importance of higher resolution data at the individual and population level and the need to understand the ecological and energetic drivers that underpin lifetime reproductive success.



How will you maximise the outputs of your work?

Communication and Engagement

presentation of the research at leading national and international conferences in a variety of fields to maximize impact.

Public Engagement

Present our findings to audiences primarily made up of school children/young people [e.g. presentations/ activities associated with animal locomotion at National Science and Engineering Week events).

Internet Resources

A website detailing the objectives of the research will be set up for access by anyone with interest, including school children and teachers. The website will contain details about the project, links to our publications, public engagement activities, conference talks, and associated media exposure. Examples of high-speed video recordings illustrating the science will be included to engage public interest and inform them of the research and its applications.

Symposium/Workshop

organized to disseminate the information acquired during the project. Leading academics will be invited from biomechanical, physiological, and ecological fields, together with representatives from research departments at NGOs such as Royal Society for the Protection of Birds and British Trust for Ornithology.

Species and numbers of animals expected to be used

- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Most birds will be trained to exercise until they can sustain locomotion for several minutes, including with the addition of external recording equipment. This may take several months.

Once the birds are trained, the birds will undergo exercise tests under a range of conditions while physiological recordings are made. Some of these recordings will be made while the bird exercises with external recording equipment attached. Other recordings will require the prior surgical implantation of devices internally, which will last approximately 2-4 hours, followed by recordings from the implanted devices once the birds has recovered from surgery, several days later. Once the recordings have been made during locomotion, some birds will be used to measure muscle contractile properties under



non- recovery anaesthesia. Birds will be killed at the end of the protocol (7-10 days after surgery).

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Some discomfort may be experienced from the presence of the internal devices and from the surgery for up to 24 hours. It is possible that some birds could lose blood during 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 species)?

Unclassified - 29%

Mild - 21%

Moderate - 50%

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 overall aims of our research are to improve our understanding of how muscles function during locomotion. This work can only be done on live, freely moving animals and could not be replaced by a non-animal method. Furthermore to develop and validate computer models of locomotion requires data on the mechanics and energetics of movement. These data do not currently exist for birds, or indeed any other experimental animal. Therefore a systematic physiological and biomechanical investigation of bird locomotion is required in which all the primary determinants and measures of movement and energy expenditure are measured.

What was your strategy for searching for non-animal alternatives?

Computational models could potentially be used to investigate the control of locomotion and the influence of the energetics of locomotion on animal behaviour.

Why were they not suitable?

A comprehensive validation of a computer model requires that a high proportion of the model inputs are measured directly so that the model represents the behaviour of a real



animal to the highest possible degree, and that a high proportion of biomechanical outputs are measured directly so that the accuracy of the model outputs can be measured directly against real data. These data do not exist for the locomotory system of birds, or indeed any other experimental animal. Although locomotion in animals has been extensively studied, no single study has measured both mechanical and energetics variables in the same species.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Power calculations were performed for each principal outcome measure to determine the minimum number of animals required. Past experience in performing each technique was used to assess the likelihood of success for each measurement, and incorporated into the calculation of animal numbers.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Online sample size calculators were used to calculate the appropriate number of animals for each part of the study together with means and standard deviations from the literature for each of the variables to be measured.

http://www.3rs-reduction.co.uk/html/6_power_and_sample_size.html
<http://www.biomath.info>

What other measures apart from good experimental design will you use to minimise numbers?

To minimise animal numbers, where feasible, multiple measurements will be made on each animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Muscle performance during locomotion will be investigated using a variety of physiological techniques that have been selected as the most suitable approaches currently available



for acquiring the data required. Since the purpose of the research is to investigate normal locomotion, it is important to select techniques that will not adversely affect locomotion.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The research must be carried out on animals capable of performing normal locomotion, and can only be done on live, freely moving animals, fully developed, adult animals.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Surgeries will be carried out by experienced (or closely supervised) individuals. Analgesics will be used post-surgery for pain relief. Post-surgery we will observe the animals for appearance and potential behaviour indicators of pain. In event of observing indicators of pain, analgesia will be used. Any animal exhibiting excessive swelling at the surgical sites will be killed humanely using a schedule 1 method.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Including, but not limited to,

On anaesthesia and surgery: Degernes, L. (2008). Anesthesia for companion birds. *Compend Contin Educ Vet.* 30:E2

On husbandry: Hawkins, P. et al. (2001). Laboratory birds: refinements in husbandry and procedures. *Laboratory Animals* 35 (Suppl. 1).

Guiding principles for preparing for and undertaking aseptic surgery. *Laboratory Animal Science Laboratory* (2017). <http://www.lasa.co.uk/wp-content/uploads/2017/04/Aseptic-surgery-final.pdf>

How will you ensure you continue to use the most refined methods during the lifetime of this project?

All project partners are already involved with 3Rs and will participate in regional NC3R events and liaise with the NC3Rs regional programme manager. In pursuing the programme of research we will implement any advances into our research programme.

Explain the choice of species and the related life stages

Birds have been selected that will sustain either exercise in the laboratory. Species choice is based on experience in previous work. The size of animals that will be used will be determined by that which is suitable for the techniques that we will use. Relatively large species are most suitable because any added recording equipment will have negligible effect on their locomotion.





170. The murine hypothalamic-pituitary axis: development and tumorigenesis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Mice	juvenile, adult, embryo, 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 is the aim of this project?

The aim of this project is to understand the mechanisms that control the development of endocrine organs: the hypothalamus and pituitary gland (HP-axis), their effect on peripheral organs and how abnormal development leads to disease such as tumour formation and endocrine deficiencies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 hypothalamus is a part of the central nervous system that controls the secretion of hormones from the pituitary gland. The pituitary gland is at the base of the skull and secretes hormones (natural signalling molecules) that control body growth, reproduction, lactation and metabolism. Together the hypothalamus and pituitary form the hypothalamo-pituitary (HP)-axis which is considered a master regulator of many physiological functions



vital for life. Among others functions, it controls reproduction, metabolism, stress response and growth. Moreover, the HP-axis regulates the secretion of hormones into the peripheral blood that control body circadian rhythms, appetite, body temperature and behaviour. Abnormal development of HP-axis, leads to multiple clinical phenotypes and severe endocrine disease affecting multiple organs. These conditions range in severity depending on which hormones are lacking; from severe short stature (due to lack of growth hormone) to multiple hormone deficiencies (with dwarfism, delayed puberty and infertility), gigantism (excess of growth hormone) due to a pituitary growth hormone secreting tumour. Importantly, the genetics underlying these conditions are poorly understood with only 5% of patients having a genetic diagnosis, whilst 95% of the patients have an unknown genetic aetiology that if known could allow for faster treatment and personalised clinical management. Understanding how the HP-axis develops in the mouse as an animal model is key to the identification of novel genes and the pathways that control their function. Moreover, the diseases at the centre of this project are very poorly understood and generating animal models of these conditions allow us to understand the pathology of these diseases, their progression and to identify possible therapeutic agents.

What outputs do you think you will see at the end of this project?

Understand the normal functioning and development of the hypothalamo-pituitary axis, but to also to identify causative role of novel genes which can be use to better diagnose these conditions, genetic counselling and personalized treatment based on the genetic abnormality. Publications and dissemination at scientific conferences. Sharing of protocols and reagents.

Most of the genetic causes conditions are not known, and these hampers the identification of treatments. Hence, knowing the genetic cause underlying these conditions we can identify molecular targets (such as Wnt antagonist) that could have therapeutic potential for HP-axis disease. Possible therapeutic treatments of pituitary tumours. Publications and dissemination of results to design medical trials.

Increase diagnosis capacity by gene discovery in the NHS.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Patients with hypothalamo-pituitary axis endocrine disease: such as growth hormone deficiency, combined hormone deficiencies, excess of hormones (growth hormone, ACTH, PRL) due to pituitary tumours, infertility, metabolic disorders will all benefit from our research. The prevalence of hypothalamo-pituitary disease in the general population is 1:1000. However the genetic underlying these conditions is poorly understood. As stated above, our research has identified genes important in the causation of hormone deficiencies (Foxa2, Tcf3) which now form part of genetic diagnostic panels. Within the life of this PPL (5 years) we will identify the function of several genes important in HP-axis development that could provide with new genetic diagnosis for congenital (present at birth) HP-axis disease.

Moreover, part of the research described in this PPL aims to identify treatments of pituitary tumours and hence results could have and impact on the management of HP-axis tumour disease.



Other researchers within related fields could benefit from the research from this PPL as protocols and reagents will be shared with the scientific community.

How will you maximise the outputs of your work?

All the results obtained from our research will be made available through scientific publications, presentation at international meetings, and local conferences.

All data will be published, even negative results on treatment, lack of specific phenotype in gene ablation experiments. This will ensure that duplication of the results is not produced unnecessary.

Protocols will be shared and mouse resources will be made available to the broader scientific community.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

We will use mice that have genes (DNA) modified in the same fashion that human disease to understand how faulty genes results in pituitary disease. Most procedures will use early stage development. When these genes are modified in some instances produce benign tumours that are poorly understood. We will use these models to understand early stages of tumor formations. We will also use these models to assess the therapeutic effect of already established compounds on the tumor growth. As part of understanding the plasticity of the HP-axis upon physiological endocrine challenges, we will use surgical methods to activate the HP-axis by removal of HP-axis target organs such as the gonads (orchidectomy and ovariectomy) and adrenal glands (adrenalectomy). To further understand how HP-axis tumour cells grow and to identify possible tumour growth reducing agents, we will use tumour growth assays by subcutaneous injections and subcutaneous tumour growth in mice. These approach will avoid stereotaxic injections of tumour cells in the brain area (hypothalamus and pituitary gland) whilst providing with good experimental model on tumour growth and assay target thereapeutic agents. This will include injections and/or administration of substances and or imagine techniques. Since the pituitary gland regulates key physiological functions such as puberty, fertility, growth, metabolism, body temperature, partuition, several studies to understand the impact of HP-axis abnormal development and tumour formation on these organs will take place.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Most of the effects on this PPL project are mild, with some protocols being moderate. Protocols: 1. Recipient embryo transfer; 2. Super-ovulation 3. Breeding and maintenance



genetically altered animals and 5. Effect of HP axis in puberty; are mild severity protocols with very few adverse effects. Only two protocols: 4. Surgical challenge of HP-axis and 6. HP-axis tumour bearing and growth assays are moderate. Each specific adverse effect is explained under protocol details section of each protocol and under Animal Experience section of each individual protocol of this PPL.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Most of the animals will have mild severity and 15% will have moderate. Although there are some experimental animals that will develop tumours, these tumours are not aggressive in nature and will be only be left to grow for short period of times and not to the point that these result in unnecessary pain.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 the molecular mechanism controlling the development of the hypothalamo-pituitary (HP)-axis and how aberrant development of this structure affects the endocrine system and can lead to tumour formation. The study of these complex interactions between developmental tissues, signalling molecules and their effect on body homeostasis cannot be studied in humans and requires animal modelling. The interaction between tissues such as the central nervous system regulating secretion of the pituitary hormones which in turn affect peripheral endocrine organs such as reproductive organs, the adrenal glands, pancreas, growth plate, breast, muscles and the thyroid.

Wherever possible we will incorporate *in vitro* models, however it is well recognised that primary pituitary cultures do not recapitulate the physiological pulsatile secretion patterns and are very difficult to maintain for long periods of time. Moreover, pituitary tumours need tumour micro-environment which is important for tumour growth and the tumour micro-environment form of fibroblast, immunocells, fat, endothelial cells is highly complex and difficult to recapitulate in culture.

Additionally, the development of the HP-axis in mouse completely recapitulates the human development, with most of the genetic pathways and signalling molecules having the same pattern of expression and paralleling the development of human HP-axis.

What was your strategy for searching for non-animal alternatives?

We have considered *in vitro* culture, and whenever possible for tumour growth *in vitro* cultures will be used derived from human tumour cells lines. However, these *in vitro*



cultures do not recapitulate the physiology of the HP-axis system. Moreover, although certain aspects of the project can be done in vitro (i.e expression of mutant proteins) others are just not feasible as the HP-axis is required for functions such as fertility and these requires a whole-animal system. In silico studies of physiological pulsatile hormonal secretion are not possible and no one has been able to recreate full pubertal or growth in silico as these requires multiorgans positive and negative feedback loops with the hypothalamus.

Why were they not suitable?

In vitro cultures do not recapitulate the physiology of the HP-axis system as this system is complex and involves crosstalk between several organs i.e hypothalamic hormones that impact on the pituitary that in turn via blood target peripheral organs such as the gonads. This multi organ-communication hormonal system has not been able to be created in a dish

Moreover, although certain aspects of the project can be done in vitro (i.e expression of mutant proteins) others are just not feasible as the HP-axis is required for functions such as fertility and these requires a whole-animal system. In silico studies of physiological pulsatile hormonal secretion are not possible and no one has been able to recreate full pubertal or growth in silico as these requires multiorgans positive and negative feedback loops with the hypothalamus.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 are and estimation of to maintain complex genetic crosses as breeding nucleus. For instance, the project uses mice that have several mutations (modifications) in different genes and hence need complex breeding schemes. To obtain the final experimental animal, 3 breeding nucleus need be maintained. The final genotype is then crossed with the Cre reporter line which gives 25% of embryos or pups with the desired genotype. Hence, a large percentage of animals is used to generate triple and quadruple transgenic to arrive to the desired experimental phenotype. We work with several genes that mainly form part of the Wnt/b-catenin pathways.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

To reduce the number of mice in this project, we will implement carefully optimised colony management and experimental design. All the genetically altered animals used in this project already exist and are in advance breeding stages in our animal facility.

To avoid surplus of animals, transgenic crosses are maintained in double homologous whenever possible to minimise the number of unwanted genotypes. We will keep stocks of frozen sperm and embryos to avoid unnecessary breeding if a line is not required for



several months. Genotypes of mice carrying fluorescent transgene can be genotype immediately after birth, preventing unnecessary weaning of unwanted genotypes.

For the disease models we aim to use the same parental mice for the first and second repeat experiment which reduces the amount of breeding pairs.

For some of the quantitative experiments sample size and power test calculation have been performed based on either pilot study data or already published results. We have used several software methods including the Experimental Design Assistant (EDA) from the NC3Rs

(<https://www.nc3rs.org.uk/experimental-design-assistant-eda>) to ensure that we use the minimum number of mice per group. Here we aim to achieve a minimum power of 0.8, assuming statistical significance of $p < 0.05$

(<https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html>).

In accordance with ARRIVE guidelines, randomisation and blinding will be implemented where appropriate to minimise experimental biases and all experiments will be documented and reported transparently (this is explained in each protocol under power test and sample size).

What other measures apart from good experimental design will you use to minimise numbers?

Efficient breeding and maintenance of the colony. Breeders will be kept up to 6 months old for females and maximum one year for males. Breeding cages will be checked regularly for their litter frequency and size. Small litters or low litter frequency will be replaced with new breeders.

Whenever possible breeding nucleus will be maintained in double homozygotes to avoid genotyping and surplus of wild type genotypes

In addition we will share tissue samples with lab members and other users interested in our transgenic lines. This importantly, allows other scientists to gather preliminary results without having to generate their own 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice have been selected because they are a well-validated experimental model for endocrine disease of the HP-axis. They are the lowest form of mammals that mimics key aspects of HP-axis pathology. Moreover, mice are the only mammals where their genomes can be modified and transgene technology works reliably. We will use the Cre/LoxP system tissue conditional approach by breeding floxed alleles with fluorescent reporters and then we will use these to be crossed with hypothalamic-pituitary specific Cre lines.



Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Because in lower vertebrates like in xenopus or zebra-fish the HP-axis and its function is completely different not only anatomically but functionally to mouse and humans . Anatomically in lower- vertebrates the hypothalamus develops in a very different way and hence genetic studies do not provide accurate picture.

However, since the best HP-axis models to understand human physiology are mice, inducible models will be used whenever possible, providing with more experimental flexibility, reducing the time window when mice display a phenotype.

All surgical procedures will be done aseptically and peroperative analgesia will be given and maintained after surgery for as long as necessary to alleviate pain. Mouse behavior and appearance will be carefully monitored for post-operative pain or discomfort.

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Refinements:

All animals are checked daily by members of our research team.

Tracking breeding performance, age of breeding nucleus, litter frequency, prompt genotyping of unwanted animals.

Use of analgesia in all surgical experiments.

Early induction of oncogenes in tissue specific manner rather than at the genomic level to ensure narrow window of tumour growth.

Early events of tumour formation without allowing for clinical sings.

We will increase the post-operative care in surgical procedures with the help of local veterinary.

In specific protocols such as Imaging techniques, we will use in central facilities.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

I regularly follow all the news on the (<https://www.nc3rs.org.uk/the-3rs> as a member) Best practices on breeding (<https://www.jax.org/jax-mice-and-services/customer-support/manuals-posters-and-guides>)

I regularly follow news/protocols from the https://ec.europa.eu/environment/chemicals/lab_animals/index_en.htm

<https://norecopa.no/prepare>



How will you ensure you continue to use the most refined methods during the lifetime of this project?

I keep informed about all news related to 3R through the web pages
<https://www.nc3rs.org.uk/the-3rs>.

I regularly attend the NC3Rs meetings organised locally, NC3Rs new letters sent regularly to my email account.

Communication with local veterinary and all members of the REDACTED facility.

Explain the choice of species and the related life stages

Mice will be used in this project. The endocrine organs in mice, especially the pituitary gland develops in a similar fashion to human, therefore they make the perfect animal model to understand endocrine disease in humans. Strains carrying specific gene modification will be studied for their effect on endocrine effects during embryonic development, postnatally for puberty both males and females, and pituitary tumour formation such adenomas and craniopharyngiomas. Pituitary tumours are benign slow growing tumours that have physiological effects as they lead to low production or over-production of one or several hormones. Hence murine models will be studied embryonically (to assess impact of mutated genes in early development) and postnatally both juvenile and adult animals.



171. The role of a newly identified calcium channel in cardiac function

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

Heart Failure, Arrhythmia, Ion Channel, Electrophysiology, Excitation Contraction Coupling

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The programme of work has three main objectives.

To define mechanistically how the newly identified calcium release channel regulates beating of the heart

To identify how the newly identified calcium release channel contributes to irregular calcium leak in the failing heart

To determine if this newly identified calcium release channel could be a new target for drug design in the fight against heart failure and fatal arrhythmias

Potential benefits likely to /derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be shortterm benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

The primary potential benefit of this project relates to new knowledge about the role of the newly identified calcium release channel in controlling the beat-to-beat of the heart and advancing our knowledge of how irregular activity of this channel can contribute to the generation of fatal arrhythmias and heart failure. The aim is to publish the findings in



academic journals where the information is likely to be of interest to pre-clinical scientists interested cardiovascular science and ion channel physiology.

There is also a secondary potential benefit which relates to the value of the results to clinicians, in particular cardiologists, and to the possibility that new molecular targets may be identified, for which new pharmaceutical products could be developed

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

We expect to use 980 GM mice during the course of this project (5 year period). We can breed from a pair of genet-ically altered mice lacking our protein of interest, meaning that all offspring will be genetically identical to their parents. Breeding appears to be normal for genetically altered mice with an average litter size of 4-6. Mice will be used at 16-20 weeks old.

By knowing the standard deviation (SD) from the mean from previous published (both from our group and others) and/or pilot experiments, the expected size of the response and the number of cells/experiments from an isolation that I can typically record data from, power calculation approaches can be adapted to give an indication of minimum required number of animals. The estimated number of animals required for each experiment is based on is the results of 2-tailed power calculations for each type of experiment. For all power calculations power value= 0.9.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Our experiments will involve the use heart tissue obtained from mice following the killing of animals via humane methods. The severity of the procedures conducted are expected to be subthreshold.

The genetically modified mice are already established in our collaborators laboratory and show no obvious sign of any adverse effects. Severity is therefore graded in the sub-threshold or mild range .This is a new line of investigation however and so during the lifetime of the study should any animals exhibit signs of pain, distress or significant ill health they will be killed humanely. All animals will be humanely killed at the end of the experiments by trained staff.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Most of the studies on the properties of this new calcium permeable channel have been initially carried out in-vitro both at the single molecule level and using a cell culture models. We have recently begun to utilise human stem-cell derived cell culture models to



investigate these basic principles. Human stem cells however are electrically different from mature heart cells. Full testing of the role of this calcium channel in regulating heart beat can therefore only be validated using a genetically altered mouse model whereby the protein is lacking. There are no other mouse models available to investigate this protein. There also remain too many unknowns for computer simulations to replace the use of animal tissue.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Our use of the in-vitro methods already discussed (single molecule electrophysiology, cell culture material and human tissue cultures) will reduce the numbers of animals required for the in-vivo investigation stage. One of the key principles of our experimental design is that the maximum amount of data should be obtained from each animal killed thus reducing animal numbers. This is facilitated through the use of isolated heart cells which last up to 4 hours. The design of individual experiments will generally involve factorial designs, which maximise the information obtained from the minimum resource. The proposed experimental designs and methods of analysis of the results have been discussed with the Statistical Services Unit.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The advantage of using a transgenic mouse model (where the protein of interest is lacking) is that it may give a more accurate result than using standard molecular methods which only reduce the amount of protein expressed. Such standard (knock-down) molecular methods have a low efficiency rate and the level of protein expression can be variable. The use of transgenic mice will therefore reduce variability and produce better scientific results. This will minimise the number of animals required over the lifetime of the project.



172. The role of DNA Damage response and replication factors in genome stability and cancer

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

During the course of the most recent licence, we generated several new DNA Damage Response (DDR) gene knockouts in mice, some of which presented with accelerated tumourigenesis, immunodeficiency, neurological problems and/or infertility. Hence, the overall aim of this project is to better define the role of DDR genes in tumorigenesis and the impact on other diseases/phenotypes including immunodeficiencies, neurological problems and/or infertility. We would like to determine which DDR genes are involved in tumorigenesis particularly and which strategies could be used to counteract the development of these 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?

The DDR is composed of multiple pathways and involves processes necessary to repair DNA lesions such as double-strand break, damaged bases, impaired replication and other genomic insults. The DDR is critical to healthy cells in order to preserve genomic stability and prevent tumor formation and other pathologies. In humans, hereditary mutations in



DDR genes are associated with a wide range of cancer susceptibility, neurodegenerative, immunodeficiency and infertility disorders. Furthermore, the DDR is often somatically deregulated in many cancers, which renders the cells more susceptible to DNA damage and makes them more reliant on other pathways for survival. Targeting DDR is one of the most exciting therapeutic strategies for treating cancer, which can be exploited to selectively induce cancer cell death. Studying the DDR in more detail may reveal unappreciated vulnerabilities that could be targeted in cancer therapy. Interrogating the DDR in animals will also improve our understanding of the roles of these processes in immunity, fertility and/or neurophysiology.

What outputs do you think you will see at the end of this project?

The focus of my lab is to understand how DDR pathways, including homologous recombination (HR), contribute to organismal homeostasis and disease. Since DDR processes are commonly inactivated in hereditary human diseases and are frequently inactivated at a somatic level in many cancers, we aim to understand how these pathways impact on human pathologies including cancer, immunodeficiency, accelerated aging and fertility. By exploiting the respective experimental strengths of *C. elegans*, frog extracts and mammalian cell culture my lab has discovered new DNA repair genes relevant to human disease. The purpose of this project is to determine how these disease relevant genes contribute to genome stability, immunodeficiency, aging and cancer using mouse models. Our work also has the potential to inform on novel opportunities for directed cancer therapy and other human syndromes affecting fertility, immunity, development which we will also explore in mice.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Our studies aim to provide fundamental new insights into mechanisms by which cells and whole organisms repair DNA damage to maintain genomic integrity and how unrepaired DNA can affect cancer development and other pathologies (immunodeficiency, infertility, accelerated aging).

The questions addressed in our studies are clinically relevant as unrepaired/mis-repaired DNA damage has been shown to be an important first step in the process of carcinogenesis and also impact on the development of germ cells and the immune system. The data generated by this project will not only improve our understanding of DNA repair mechanisms and how this affects organismal homeostasis but will also inform on the development of targeted therapeutics. Only by inducing dysregulation of DNA damage repair pathways in vivo will we be able to uncover their roles in carcinogenesis and/or organismal physiology.

How will you maximise the outputs of your work?

Findings will be made available to the broader scientific community through publication in peer-reviewed journals and presentations in scientific conferences. Data will also be deposited onto publicly accessible databases. Newly created transgenic animals will be distributed widely to the scientific community as these lines generated under the authority of this Project Licence will be very valuable to the research community.

Species and numbers of animals expected to be used



- Mice: 15200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Mice used will be generated to create conditional knockout, knockin, conditional over-expressing and/or BAC transgenic mice for genes we have previously implicated in the maintenance of genome stability (Protocol 1 and 2) or will be provided by other projects with authority to breed, maintain and/or supply transgenic mice.

We will investigate the relationship and genetic interactions between the resulting mice and other mutant mice that exhibit genome instability phenotypes and/or meiotic defects (Protocol 3, 4, 8, 9).

We will also characterise the tumour predisposition, neurological, immunological and/or meiotic phenotype of these mice (Protocol 3, 4, 5, 6, 7, 8, 9).

The principle aim of objectives 1-3 will be the detailed examination of tumour incidence, meiotic, immune-deficiency or neurodegenerative phenotypes in mice mutated in novel genome maintenance genes. The significance of tumour predisposition, meiotic, immune-deficiency or neurological phenotypes conferred by a particular genetic background will be judged by comparison to established mice (Protocol 3), which should show clear statistically significant difference from controls (ANOVA). Tumour predisposition will also be assessed by treating mutant mice with gene inducers or modifiers, DNA damaging agents, carcinogens, tumour promotor (Protocol 3) or by injection of cells mutated for DNA damage response genes (as for example ALC1, Rad51, WRNIP) into nude mice (Protocol 4, 5, 6, 7). These objectives can only be addressed using whole animal models, as modelling tumour development to this level of detail is not possible in vitro. The minimum number of animals will be used and all suffering kept to the lowest possible level by ensuring that animals bearing tumours are sacrificed before the tumours reach a size that is likely to cause distress.

In general, determining tumour predisposition in mice bearing a mutation in DNA Damage response genes will be fulfilled by 2 complementary ways: first, we will monitor mice in aging cohorts by performing a daily health check (clinical examination, abdominal palpation and imaging if necessary) (Protocol 3). Second, we will use a small cohort of animals, which will be treated with chemicals and notably tumour inducers/promoters and/or carcinogens (Protocol 7, 8). These 2 complementary ways should allow us to demonstrate if a particular DNA damage response gene has a role in tumour formation. Chemical carcinogenesis is one of the best-established in vivo models for the study of tumour development and for evaluating tumour initiation, promotion and progression (Protocol 8). Well-established methods also require use of a procarcinogen such as diethylnitrosamine in combination with a high fat diet to allow tumour development (Protocol 7). The high fat diet will also induce obesity, which will be used as a tumour promotor. The quantities and frequencies of the chemical used should result in no more than transient discomfort and no lasting harm. Carcinogenesis protocols (chemical or UV) have been developed for the study of tumours in mice (i.e., tumour incidence, latency,



multiplicity and progression). In complete carcinogenesis protocols, tumour development occurs either after the administration of a single high dose (or repeated applications of a lower dose) of a carcinogen or by repeated low dose exposure to ultraviolet (UV) light. UV/X-irradiation will also be used as a potential in vivo inducer of DNA damage that will in a time dependent manner induce tumour formation. Mutant animals as well as controls will be subjected to single or multiple doses of either UV or X-irradiation to allow us to determine the role of specific DNA damage response genes in DNA repair (Protocol 3, 4, 5, 6, 7, 8, 9).

Non-invasive imaging will be employed in Protocol 3, 4, 5, 6, 7, 8 to ensure that we can detect tumour(s) arising in mice as soon as possible and minimize animal suffering. We will use different types of imaging such as bioluminescence or fluorescence imaging to monitor tumour formation and growth (when for example using B16F10 cells expressing a fluorescent or bioluminescent transgene: B16-F10- Luc or B16-F10-RFP). In some cases, we may want to follow tumour progression by non invasive MRI or microCT. All imaging will be performed by experienced people working at the REDACTED imaging facility.

Clinical examination, abdominal palpations and imaging will be used to assess and confirm tumour presence in our animals. It will allow us to limit tumour burden to a minimum.

We will also assess neurological problems by employing SHIRPA behavioural tests in protocol 3.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

An important aspect of our research is to monitor animals that have genetic modifications throughout the term of their natural lifespan (up to 24 months) in order to study and assess their phenotype (Protocol 3). Humane endpoints for mice used in such longevity studies have rarely been addressed, despite the expectation that health problems will become more common as the mice age. Natural and spontaneous death can occur without being linked to any genotype. In rare cases, aged animals can die without having shown any prior signs of illness or deterioration in health. We will monitor animals at risk with greater than usual frequency and any changes in their health status will be accurately recorded in a form specifically designed for animals at risk of death and archived. If animals are found dead without having shown prior clinical signs, post-mortem investigation will aid determination of cause of death and this data will then be analysed as soon as possible so that early euthanasia can be performed on other animals to promote their wellbeing as well as facilitating collection of valuable samples and measurements.

Mice on protocol 4, 5, 6, 7, and 8 will develop tumours as adverse effects. For mice used under protocol 4, 5, 6, tumours development will generally be monitored twice a week by either calliper measurement and/or imaging if possible. Animals developing tumours larger than 1.2cm or 1.5cm will be humanely sacrificed. These experiments should be short term experiment that may be no longer than 3 months. Mice under protocol 7 and 8 should also develop either external (protocol 7) or internal (protocol 8) tumours that will be monitored frequently. These protocols may last up to a year to follow induced tumours development in a genetically altered animal.

In this project, substance administration should not cause adverse effects that are longer than temporary and transitory as we will for most of them use doses that have already been described by the scientific community.



Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Only mice will be used in this project.

A high proportion of mice (75%) should develop phenotypes of moderate severity as a result of tumours development, immunodeficiency and/or neurological problems. A subset of these mice will also be used for tissue harvest and/or cells production.

25% will have a mild severity and the remaining would be subthreshold.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 and those in the published literature on the DNA damage repair pathways and other DDR genes are suggestive of roles in maintenance of genome stability and tumour avoidance; Brca1, Rif1, Rtel1 and ALC1 have been observed to be either amplified or inactivated in tumours depending on context. What remains unclear is if these changes are a cause or a consequence of tumorigenesis. Animal experiments remain the only definitive test of whether inactivation/over-expression of these genes is relevant for tumour formation. These issues can only be addressed using whole animal models, as modelling tumour development to this level of detail is not possible in vitro. The use of mice offers an unparalleled level of genetic tractability in an organism that develops cancers, immune-deficiency or neurological logical problems that are comparable to those occurring in humans.

3D model such as cultured organoids are good model to answer a specific question but the relevance at the human level is very limited as it does not include the specific environment in which a tumour is growing. Only a genetically altered mouse model will be reproducing the same environment than the one observed in a human body. Moreover, genetic drift in organoids culture may happen quicker and won't be as well controlled as it is in a mouse model.

What was your strategy for searching for non-animal alternatives?

All of our proposed studies aim to build on extensive groundwork in model organisms, mammalian cell culture and during the last 5 years in mouse models. The objectives outlined in this application represent the next and most pertinent step forward in our mouse studies, which is likely to provide novel insights into the impact of these factors on organismal biology and disease. We are currently using a multi system approach to answer questions addressed in our studies. For example, we use the C.elegans worm



model as it is a very easy organism to genetically manipulate. It makes the study of synthetic lethality very accessible due to its quick reproduction and also is a very good model for studying meiosis as all steps of it are represented in the germline. We also use the frog model *X.laevis* to study replication-coupled DNA repair. One of our main tools is the use of mammalian cell culture, which was recently revolutionised by the discovery of CRISPR. Generation of genetically altered mouse or human cell lines is proving to be easier and quicker with CRISPR techniques and these cell lines can be used to do targeted proteomic screens that will give us more insight on molecular mechanisms that we will be able to study more in depth at the level of a whole organism. Only by inducing dysregulation of DNA damage repair pathways in vivo will we be able to uncover their roles in carcinogenesis and/or organismal physiology.

Why were they not suitable?

The proposed experiments simply cannot be addressed in frogs, worms or other model organisms, as they do not develop tumours, immune-deficiency or neurological problems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For obtaining statistically significant and reproducible results, we will include a positive and a negative control group which will give validity to the ongoing experiment. Wherever possible, power calculation with a power of 80-90% and a type I error rate of 5% will be used to more accurately estimate the number of animals needed to ensure a statistically significant outcome and avoid unnecessary experiment duplication.

In all in vivo experiment, the number of mice required for a specific experiment is based on 3 criteria:

The number of animals for each set of experiments

The number of experiments (needs to be perform 2 independent times)

Pilot studies and previous experience performing a similar experiment

Where necessary we will also consult the bioinformatics and biostatistics group at the REDACTED during the experimental design stage to ensure that the appropriate numbers of mice are used per experiment.

All experiments/procedures notably tumour development measurement will be performed limiting the animals' number to the minimum required for a valid scientific outcome.

A specific subset/cohort of animals will be used under each protocol. We will also use animals to harvest tissue and/or produce cells (MEFs, ES cells). Some experiments could require up to five genetic modifications to be bred into one animal, which would require extensive breeding programmes.



What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Mouse breeding experiments will be planned in consultation with the REDACTED BRF and the basic principles of mouse breeding will be adhered to and gestation times, weaning age and litter sizes used to calculate the numbers of mice required to optimise the size of the colony. REDACTED will be routinely maintained by keeping 2 breeding pairs. For crosses under analysis for specific phenotypes it is likely that 5-6 breeding pairs will be maintained for the duration of the experiment. However, every effort will be made to reduce the number of animals required for experiments, always keeping in mind that enough animals are used not to prejudice the generation of statistically relevant results. For exploratory (pilot) experiments the least number of animals will be used (usually 4-6 per genotype/experimental group) to provide a reasonable estimate of the numbers of animals needed to produce statistically relevant information in subsequent quantitative experiments. Where possible we will use data from published experiments to provide an estimate of the sample size.

Where necessary we will also consult the bioinformatics and biostatistics group at the REDACTED during the experimental design stage to ensure that the appropriate numbers of mice are used per experiment. We will also use the Experimental Design Assistant from NC3R to help us design our experiments (ensure the use of minimum number of animals consistent with our scientific objectives).

For multiple genetic modification, we will investigate alternative methodologies to enable reduced numbers of animals to be used. This will be to use viral delivery of the expression modifying genes CRE and tetracycline activator via different routes (intratracheal, intraperitoneal, topical application). This would require two less genetic modifications to be present in the mice, so would greatly reduce the size of the breeding programme.

We will also reduce numbers of mice by cryopreserving sperm or embryos with the help of the Genetic Modification Services of the REDACTED.

What other measures apart from good experimental design will you use to minimise numbers?

We are always looking for ways to reduce the number of animals that we produce. Breeding efficiency is key and for strains that are known to have low breeding efficiency, we feed females with baby milk mash. This allow us to use less mice for breeding and also increases the chance to have a higher number of embryos/pups in some of our C57BL6 mice. Moreover, most of our xenograft studies start with pilot experiment using small animal cohorts that lead to a refinement in term of cell number injected, and/or doses used for specific compounds and give us insight into experiment duration; and where possible we will use a single group of animals as a control for several treatment groups.

Other ways in which we will aim to reduce mouse numbers will include freezing down transgenic lines; deriving cell lines from mice for specific cell culture experiments; providing tissues from our mice to other labs.

The breeding of genetically altered mice will be reduced through collaborative access to strains.

Refinement



Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

Mice will be our model of choice during this project. The use of mice offers an unparalleled level of genetic tractability in an organism that develops cancers, immune-deficiency or neurological problems that are comparable to those occurring in humans. This is particularly relevant to our objective where we are attempting to provide evidence that DNA damage response factors are involved in specific diseases. We will first attempt to test our hypothesis in tissue culture with cells derived from these animals, but ultimately, it is experiments within the animal that will have the biggest impact on human disease. We will minimise the numbers of animal used and minimise the suffering of animals during experimental procedures.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice and Human are biologically and genetically very similar. By using mice, we ensure that we can study the effect of a single specific genetic alteration (for example found in human breast cancer or any other disease that have a deregulated DNA Damage repair pathway) in a very controlled organism. Moreover, spontaneous tumourigenesis resembling human tumorigenesis cannot be reproduced in other organisms (flies, worm, frog, cell culture) than a mouse model, as they do not develop tumours, immune-deficiency or neurological problems. Where possible experiments on animals will be replaced with experiments on cultured cells, *C.elegans* and *X.laevis* as outlined above. We will first attempt to test our hypothesis in tissue culture with cells derived from these animals, but ultimately, it is experiments within the animal that will have the biggest impact on human disease and new therapy findings. We will minimise the numbers of animal used and minimise the suffering of animals during experimental procedures.

What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We will monitor animals at risk with greater than usual frequency and any changes in their health status will be accurately recorded in a form specifically designed for animals at risk of death and archived. If animals are found dead without having shown prior clinical signs, post-mortem investigation will aid determination of cause of death and this data will then be analysed as soon as possible so that early euthanasia can be performed on other animals to promote their wellbeing as well as facilitating collection of valuable samples and measurements. We will use palpation and imaging in order to limit adverse effects due to tumour development.

The weight of the animals going through a procedure will be monitored closely to ensure weight is stably increasing and is not affected by any procedure that a mouse went through.



Most of the protocols that we are using in this project are well established protocols that have been widely used by the scientific community. We will follow them closely by administering the recommended concentration of substances (such as chemical carcinogen, tumours initiator and promotor, IR/UV doses, antigen concentration).

To improve our breeding efficiency with difficult to breed mice, animals will be fed with mashed food supplemented with milk powder.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

FELASA “Working Group on Pain and distress” and NCRI guidelines will be employed to aid in the assessment of pain and distress and used to determine the earliest endpoint possible to allow a valid scientific outcome.

When assessing tumour burden, the NCRI Guidelines for the Welfare and Use of Animals in Cancer Research will be followed.

In addition, we will follow the PREPARE guidelines when designing experiments and ARRIVE guidelines when publishing to allow better reproducibility and avoid work duplications.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We are currently subscribing to the NC3R monthly newsletter which gives us information on any techniques’ improvement and 3R advances. We are also in relation with the regional programme manager for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) who can give us advice and support on the 3Rs for our project.

Explain the choice of species and the related life stages

The use of mice offers an unparalleled level of genetic tractability in an organism that develops cancers, immune-deficiency or neurological problems (such as ataxia, impaired balance, coordination and/or behaviour) that are comparable to those occurring in humans. This is particularly relevant to our objective where we are attempting to provide evidence that DNA damage response factors are involved in specific diseases (any types of cancer, Riddle syndrome, Hoyeraal-Hreidarsson syndrome, FILS). We will first attempt to test our hypothesis in tissue culture with cells derived from these animals, but ultimately, it is experiments within the animal that will have the biggest impact on how human diseases such as cancer develop. The proposed experiments simply cannot be addressed in flies, worms or other model organisms, as they do not develop tumours, immune-deficiency or neurological problems. Where possible experiments on animals will be replaced with experiments on cultured cells as outlined above. However, in cases where this is not applicable, we will adhere to the three Rs (Replacement, Reduction and Refinement) to minimise the numbers of animal used and minimise the suffering of animals during experimental procedures.



173. The role of muscle stem cells in health and disease

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.

Key words

Skeletal Muscle, Muscle stem cell, Muscle Cancer, Muscular Dystrophy, Facioscapulohumeral muscular dystrophy

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

Skeletal muscle is able to repair and regenerate because of resident stem cell. However, as people get older, this repair becomes less efficient, leading to muscle weakness and wasting. Repair also fails in some diseases, again leading to weakness and wasting. Muscle stem cells can also become deregulated, which can lead them to forming cancerous tumours. We investigate the regulation of muscle stem cells to understand how they are controlled in healthy muscle, and what goes wrong in disease. This research will inform the development of therapies to treat such muscle disease and possibly ameliorate some of the effects of aging on muscle function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

Understanding how muscle stem cells are regulated in healthy muscle, and what goes wrong in disease and cancer, will lead to potential therapies to ameliorate this deterioration in muscle stem cell function.

Species and numbers of animals expected to be used



What types and approximate numbers of animals will you use over the course of this project?

We use mouse, and estimate that we will use approximately 2600 over the five year duration of this licence.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

To understand how muscle repairs itself after injury, we need to damage specific muscles while the mouse is anaesthetised and then examine the repair process. Since we only injury one muscle, the vast majority of the limb musculature is unaffected, and so gross limb function remains overtly normal. After the experiments, the mice will be humanely killed and their tissues removed for analysis.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Obtaining muscle cells directly from patients for every experimental procedure is impractical. We will use human cell lines derived from healthy and patients, together with mouse cell lines where available.

The most effective model for routinely obtaining stem cells however, is mouse, and mice have been generated that model many human diseases. These cells form muscle fibres in tissue culture, but do not mature without nerves, blood vessels and connective tissue. This is why we have to examine muscle stem cells directly from the mouse.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Our extensive use of cell lines limits the numbers of animals required. Where possible we initiate regeneration in one leg and use the other as the control. Mice will be bred so that we get the maximum progeny with the correct genotype that we need. Pilot studies will be performed to determine the lowest effective dose with new reagents and estimate sample size. The aim is to use the minimum number of animals to obtain statistically significant results and thus be able to determine any difference between experimental and control group.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.



Our standard non-human model is the mouse, a mammal with a short gestation period and the ability to be relatively easily genetically altered, thus there are many mouse models available. Mouse is also a good model to study regulation of muscle regeneration since there are many useful reagents available. Furthermore, human and mouse cells will fuse together to form mosaic human/mouse muscle fibres in culture, showing that many of the signalling molecules are able to interact. Importantly, many of the key genes involved are conserved in man. We would therefore expect findings on mouse satellite cell regulation, muscle regeneration, disease progression and therapeutic intervention to be broadly applicable to human.

Good animal husbandry practice is used, and advice sought where necessary. There is a regular use of pain-killers after surgery, and steps like providing moistened food on the floor on the cage used where needed. For transgenic and genetically altered mice, inducible constructs will be used whenever possible, so there should be no phenotype until candidate gene expression or deletion is induced. By targeting the induction to muscle for example, we should avoid any potentially damaging effects of systemic expression. We do not undertake any procedures that are classified as severe.



174. The role of RAS and RHO-like GTPases and their regulators in lung 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

No answer provided

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 is the aim of this project?

To identify changes in signalling genes in lung cancer cells, and to relate these changes to the ability of lung cancer cells to multiply and spread. Ultimately, to use this knowledge to develop new treatments for patients with lung 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?

Lung cancer accounts for nearly a quarter of UK cancer deaths. Its most common histological subtype is adenocarcinoma (50-55% of patients), of which the most common driver mutation is in the small GTPase KRAS (a cell signalling gene) (~30% internationally). Genetic data has altered the classification of non-small cell lung cancer (NSCLC) over the past 10 years, highlighting specific genetic alterations (in signalling genes), which have improved prognosis and are susceptible to targeted therapies. However, no targeted therapies exist for lung cancers caused by the KRAS mutant gene



(KRASm). There are three main variations of the KRAS gene in NSCLC. Breakthroughs in targeting these mutant KRAS variants have the potential to offer similar, if not greater, clinical impact to current targeting therapies against other signalling genes. Studies increasingly support the hypothesis that distinct KRAS activating gene variations may promote lung cancer formation via different mechanisms and this may be exploited for variation-specific treatment interventions. We therefore aim to characterise the relative roles of KRASm gene variations in NSCLC tumourigenesis using cell as well as mouse studies. In doing this, our goal is to further refine classification of NSCLC providing evidence to support the subdivision of KRASm lung cancer resulting in more personalised medicine.

It is well established that the enzyme RAC is required for the formation and growth of many types of tumours including KRAS mutant NSCLC. Our recent cell derived data have also shown that RAC and certain of its activators are required for survival of small cell lung cancer cells (SCLC), a highly metastatic subtype of lung cancer accounting for around 15% of all lung cancers. RAC is a member of a family of related molecules called RHO GTPases. These molecules and their activators and regulators are important not only for the survival and growth of tumour cells, but also for the metastatic (cancer spread) process. Metastasis is a challenging clinical problem and is the cause of most cancer deaths. The involvement of RHO GTPase signalling in the survival, growth as well as metastasis of cancer cells, means that RHO GTPases and their many regulators are candidate therapeutic targets for cancer growth and spreading.

What outputs do you think you will see at the end of this project?

The primary output of this project is data / information that advances our mechanistic knowledge of how different mutant variations of RAS and RHO GTPases drive the development and progression of lung cancer. Our findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings.

The expected benefits of the work can be summarised as follows:

Knowledge of the pathways by which the different KRAS variants and associated biochemical pathways present in NSCLC control lung tumour formation and progression

Understanding the role and mechanism by which members of the RHO GTPase family of enzymes regulate NSCLC and SCLC

By integrating the knowledge gained from the above research, we aim to identify key therapeutic targets for future hypothesis driven therapeutic intervention

Test novel therapeutic agents currently investigated in clinical trials and their potential mechanism of loss of effectiveness due to drug resistance

Publication in high quality journals and presentation at conferences to share the work with the wider scientific community

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?



We anticipate that a large part of benefits stated above would be seen within the 5 year duration of the project. The main beneficiaries of these will be other scientists, health professionals as well as pharmaceutical companies working on lung cancer. We anticipate that patients won't be able to benefit from this work within the time frame of this project licence, but might benefit in the next 10 years. Patient benefit might be via better classifying lung cancer and understanding better which patients are likely to respond to current treatments as well as by developing new compounds.

How will you maximise the outputs of your work?

Our findings will be made available to other scientists through collaborations, publication in high quality journals and presentations at scientific conferences and meetings. Our Institute has a policy of ensuring that all publications from Institute scientists are available on free access to all.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Some mice (bred under other Project Licences) harbour genetic modifications that predispose them to the development of lung cancers when exposed to an appropriate inducing agent. Furthermore, some genetic modifications allow us to track specific cells within the tumour (such as T cells). In other mice, which are bred specifically to tolerate human cells, tumours will be implanted under the skin of the mouse for ease of monitoring or into the lung so that cells are placed in their physiological environment. Mouse cancer cell lines can be transplanted into mice sharing the same genetic background (so called syngeneic mice) without rejection. 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 for superficial tumours, or by imaging methods for internal tumours. Mice with tumours will be monitored daily. For some procedures that involve surgery under general anaesthesia, such as implanting human tumour fragments or removing a primary tumour in order for a secondary tumour to grow, we will administer pain killers and monitor the mice closely during recovery from anaesthesia.

Some mice may have either potential novel therapeutic agents, existing clinical agents or dummy-drug administered by a variety of routes, but usually either by mouth, or by injection either under the skin or into the abdomen to study the effects on tumour growth and / or tumour composition. Mice may be studied for up to 200 days after a period of therapeutic agent treatment for tumour growth. The mice will also have blood samples taken either from the tail vein or by sampling from a heart chamber under anaesthesia (in which case the animal does not regain consciousness before humane termination). Occasionally mice may be administered organ preservative whilst under non-recovery anaesthesia to allow us to undertake investigations on slices of selected organs observed under a microscope.



Mice will be group housed in ventilated cages which have their environment enhanced with items such as tunnels, houses, nesting material and gnawing blocks.

At the end of any protocol mice will be humanely killed.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The impact of tumourigenic mutations are not expected to cause any adverse effects per se as these in most cases only manifest following inhalation of inducing agents. Following inhalation of inducing agents mice carrying tumourigenic mutations are expected to have lung tumours. It is possible that the tumour growth might affect normal physiological functions (such as eating, locomotion or breathing). However, mice will be observed daily and any side effect that cannot be managed satisfactorily will result in humane culling of the animal.

Injections would only cause very transient pain.

After surgical procedures we will monitor mice for signs of pain and administer effective pain relief for as long as it is required.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

The vast majority of mice are only expected to experience the mildest clinical symptoms due to tumour growth before they are humanely sacrificed. Additionally, some mice will experience the discomfort of repeated (daily) injections of therapeutic agents or oral delivery with a specialist tube. We will aim to utilise the least stressful route of administration wherever possible.

A minority of mice will undergo surgery and these will be anaesthetised for the operation and receive pain killer post-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.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

While valuable studies of human cancer are performed using tumour material and cell lines derived from both mice and human samples, the mechanistic understanding of



cancer development and spread requires use of living animals. In particular, cancer development and spread involves a plethora of interactions between cancer cells and their surrounding host and their behaviour is governed by multiple signals originating from both their immediate neighbours and from distant tissues.

Genetically altered mouse models have been engineered to develop cancers, which accurately mimic their human counterparts. These models can be used to test the effectiveness of novel cancer therapeutics that have been developed for humans as there is a big similarity between human and mouse proteins. Mice cannot be replaced by different animal models such as zebrafish or insects which remain far less complex than their murine counterparts and for the purpose of our work do not have lungs.

What was your strategy for searching for non-animal alternatives?

We will use a variety of cell-based approaches to investigate how manipulation of RAS and signalling pathways of interest alter cell behaviour in cultured lung cancer cells prior to undertaking in vivo studies. We also make use of human lung cancer specimens and sequencing data to generate further evidence in support of our hypotheses and to check that findings are relevant to clinical samples.

Why were they not suitable?

The study of cells in culture (in vitro) provides us with clues on the mechanisms of cellular processes in a simple and valuable context, which allows the establishment of hypotheses regarding the function of cells in a living animal. However, these systems do not recapitulate the complex cellular interactions described above.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The overall aim will be to generate models whereby a measurable effect e.g. reduction in tumour volume or tumour incidence following manipulation of a gene of interest or treatment with a drug can be determined using a minimal number of animals.

Data available from the literature or from pilot studies are used to determine an appropriate sample size for the definitive experiment. In general, we will use a sample size capable of detecting a 40% practical difference with 80% power and 95% confidence.

Based on past experience, group sizes of between 10 and 30 animals (dependent on the readout; fewer for transplanted tumours compared to spontaneous tumours in genetically modified mice) per experimental group suffice. However, for an experiment to be well controlled and meaningful, we may include more than one experimental group. For instance, in implantation experiments where we deplete a gene in cells, we will use two independent reagents targeting the gene as well as a control. Moreover, we would typically examine more than one model cell line. Likewise, we may use several doses of a drug, or several different drugs or drug combinations to test a theory. Considering power, the



number of experimental groups, and the number of genes and drug targets we are interested in, we have then estimated the total number of mice to be used over the licence lifetime.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

For genetic mouse models, efficient breeding strategy will minimise the number of mice used to obtain the desired genotype.

Experiments will be appropriately controlled and mice of the same age, genetic background and source will be used to reduce the variability of results and to produce highly consistent data. Wherever possible and appropriate, a single group of animals will serve as a control for more than one experimental groups.

We will be conducting and recording our experiments to be able to publish our results following the ARRIVE guidelines [<https://www.nc3rs.org.uk/arrive-guidelines>] and will use randomisation, blinding etc. where appropriate so as to minimise biases. Furthermore, additional resources may be used to aid experimental design such as the NC3Rs experimental design assistant tool

(<https://www.nc3rs.org.uk/experimental-design-assistant-eda>).

What other measures apart from good experimental design will you use to minimise numbers?

Pilot studies will be performed if applicable and, after analysis of the results, group sizes for subsequent experiments will be determined based upon these data. As far as possible, multiple parameters will be evaluated in a single mouse. Live imaging of the same animal at multiple time points also greatly reduces the numbers 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The mouse is the lowest vertebrate that offers an in vivo situation (anatomy, physiology, metabolism) relevant to human cancer and that can be manipulated in a manner that will generate data relevant for the treatment of human cancer. As such, the mouse is the most appropriate animal model to achieve the stated objectives. Moreover, techniques required for the proposed types of analyses are established for studies using mice making predictions of possible adverse effects more reliable.

We will use mice that have been altered genetically to either allow specific tracking of cells or which are predisposed to lung cancer conditionally upon treatment with inducing agents. Tissue specific and inducible gene alteration will reduce the potential burden of genetic



loss in the whole organism. The vast experience of animal technologists within our animal facility will be harnessed to minimize potential suffering by regular monitoring of tumour size and potential impact on the general health status of the mice. By responsibly considering the adverse effects associated with the regulated procedures, mechanisms are in place to minimise these (e.g. appropriate analgesic regimes for pain relief).

To reduce any suffering of tumour bearing mice, they will be killed humanely as soon as tumour formation is sufficient to yield satisfactory data and always before they become moribund, manifest severe pain or lose significant weight (all of which are closely monitored).

For any new procedure, we will seek expert advice and follow the most refined techniques available, experimenting at first with a limited number of mice.

For any inoculation/transplantation procedure we will follow the route that causes the minimal burden on the animal's well-being.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Less sentient animals do not have lungs. Mouse is far more similar to humans than other animals having lungs e.g. birds or reptiles and this is critical both for using reagents like drugs developed for human targets and for translating findings to the clinic. Furthermore, cancers develop over many weeks to months, so use of terminally anaesthetised animals or immature animals is not practicable. Also immature mice lack a functional immune system which is desirable in cancer research.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Assessment of the size of superficial tumours would usually be by callipers (usually of two diameters at right angles). The total tumour burden should not normally exceed 1500 mm³ as calculated by the formula: tumour volume = $\frac{1}{2}$ (length x width x 2). Sub-cutaneous tumours will be measured at appropriate intervals (usually once a week using callipers). If a tumour reaches approximately 2/3 of the maximum permissible volume, it will be measured more frequently.

Additionally, superficial tumours will be monitored for signs of local inflammation, irritation, or pinpoint scabbing to an ulcerated state. Animals with ulcerated tumours will be cared for according to the best practice at our Institute. Wherever relevant, animals will be provided with analgesia as detailed in the relevant Protocol to control adverse symptoms associated with the treatment.

For non-superficial lung tumours imaging will be used to monitor the disease burden alongside measures of the health status of the animals (e.g. respiratory rate, lethargy).

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

Relevant published literature will be used as template for experimental design and decision making (Workman et al., 2010. Guidelines for the welfare and use of animals in cancer research. BJC, 102, 1555-1577).



We will follow guidelines of good practice [Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, et al. British Journal of Cancer, 102:1555-77 (2010)] 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.

Guidelines for Body condition score. [Ullman-Cullere, Lab Anim Sci. 1999 Jun;49(3):319-23]

How will you ensure you continue to use the most refined methods during the lifetime of this project?

By reading 3Rs literature and participating in 3Rs workshops locally and nationally. Through discussing refinements with our NACWO, NVS and HO inspector.

Explain the choice of species and the related life stages

Mice are more comparable to humans than other animal model systems (fish, invertebrates) in pathophysiology and show higher similarity of genetic and protein sequences. This is important as we intend to use reagents such as small molecule inhibitors and antibodies that have been developed to target human proteins.

Moreover, non-protected species and less sentient species (fish and invertebrates) do not have lungs so we would be unable to use them for animal models of lung cancer. Embryonic stages would not provide us with a sufficient window to follow tumour development and besides it is not feasible to perform the desired interventions in embryos (such as inhalation of activating agents). Therefore adult mice are to be used.





175. The use of novel patient derived and mouse models to develop better treatments for advanced resistant cancer

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants.

Key words

Prostate Cancer, Metastasis, Resistance, New Treatments

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

Prostate cancer is the most commonly diagnosed cancer in men and a common cause of male cancer-related death. Treatment of prostate cancer has improved over the last decade, but while the disease often responds to treatment initially it is common for it to become resistant and reoccur. The re-occurring disease is always more aggressive than the initial tumour and is accompanied by painful metastasis often to the bone and the cancer has been proven to find various ways to escape the selective pressure of medicalisation given to the patient. In order to develop therapies that prevent or cure the advanced state, it is extremely important to understand how prostate cancers become drug resistant and what type of strategy might help. For this we need cells to be grown in petri dishes to select the best candidates for new treatments and in mice to prove or disprove that the selected drugs are suitable for treating cancer in a living organism. Current prostate cancer culture models used to study the disease and to develop novel treatment strategies for lethal prostate cancer have been generated more than two decades ago and do no longer adequately resemble the human disease. Better models to develop new advanced therapeutic approaches are therefore urgently needed. Scientist all around the world including ours have been trying to develop new models with some success, but not enough to cover the full spectrum of different resistance tumour types we are currently facing in the clinic. Our group has recently developed a very successful strategy to make small tumour pieces from patient biopsies grow in mice first, in order to



get enough tumours to then develop cell lines growing in petri dishes and mice. So far, we have developed 4 new models and that cover some but by far not all types of resistant prostate cancer. These have been used in various tests and have greatly contributed to the field already. We now would like to apply this successful strategy to cover a broader range of resistance mechanism for the benefit of patients suffering from other types of advanced prostate 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.

What are the potential benefits that will derive from this project?

We plan to develop better in vitro and in vitro models of prostate cancer grown from small tumour pieces called 'biopsies' taken from patients suffering from treatment-resistant prostate cancer. We will use these models to better understand how these cancers become resistant and to develop strategies to reverse resistance and cure patients suffering from the at the moment un-curable disease stage. We will furthermore develop mice that do not express or overexpress genes that have been identified as new drug targets and cross them with mice that have been engineered to develop prostate cancer. This will help us to better understand the importance of these genes for prostate cancer growth and survival and will validate them as potential drug targets in order to then develop new drugs and therapeutic strategies to better treat the disease.

Both types of models will help us to better understand the biology of these tumour types and how to best address them therapeutically. This will greatly add to the gain of knowledge in the field as we will publish our results in peer reviewed journals to make our findings accessible to other scientists around the world and will be extremely useful for testing new therapeutic approaches for multi-resistant prostate cancers in culture as well as in living animals.

Species and numbers of animals expected to be used What types and approximate numbers of animals will you use over the course of this project?

We will use no more than 13,000 mice over the whole five-year period.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

All of our experiments will ultimately lead to mice that will suffer from prostate cancer. We will therefore take extra care for them and our trained personal will monitor them closely under the supervision of a veterinarian trained in the field of cancer related animal studies. Mice will be checked at least four times per week for signs of tumour growth and distress, and will be weighted once per week. Tumours will be grown up to a size limit in order to ensure they do not interfere with normal activities and behaviour, and mice will be killed



using a humane method as soon as their tumour reaches the size limit or their behaviour is affected.

The mice used to implant the tumours need to be special immune deficient mice, some of them need to be irradiated in order to suppress immune response or to treat the cancer after implantation. To prevent infections, the mice will be kept under strict aseptic conditions with sterilised bedding, water, and food and supplied in cages with filters to remove pathogens from the breathing air. Immune deficient mice will be monitored daily and mice and environment will be regularly tested for potential pathogens that could endanger wellbeing of the colony.

Implantation of biopsy requires surgery that will be under aseptic conditions, general anaesthesia and post-operative analgesia. After the procedure, mice will be observed carefully and painkillers or antibiotics will be given if necessary. In order to minimise distress, we will use non-invasive methods to monitor tumour growth such as callipers, ultrasound, NMR or PET, some of which need general anaesthesia. Treatment will be applied by injection (under the skin, or into the abdomen) or given directly into the stomach using a needle or tube inserted into the mouth which will cause some distress but is not likely to cause long lasting discomfort. Some of the treatments given will cause side effects such as weight loss. Radiation can potentially cause skin burn, or weakness. We will closely monitor the mice and humanely kill them if we can't treat adverse effects with the measures described above and are concerned about the severity of distress they are experiencing, or otherwise at the end of the experiment.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Our group and others have greatly improved methods to culture of tumours pieces in culture (known as tumour organoids) for use in drug testing. We plan to use these methods to test novel therapeutic approaches in culture first, which will markedly reduce the number of mice needed for experiments. Although it is possible to use the biopsy directly in culture, this approach is limited as patient tumour biopsy is small so we can only use it for a small number of experiments. We therefore plan to increase the number of possible tests in order to compare and optimise treatments by growing the small biopsy into little larger tumours by implanting it under the skin of a small number of mice at a site and up to a size which will have the lowest impact possible on the wellbeing of the mouse. These tests will greatly decrease the number of animals needed to understand the tumour biology and to identify the best strategy to treat tumours that are resistant to established treatments, however, to ultimately test if such a treatment is working in a living tumour and before it can responsibly used in patients, an animal experiment is inevitable.

Reduction

Explain how you will assure the use of minimum numbers of animals.

We will use inbred mouse strains with very low degrees of genetic variation to maximise those that will be useful for experiments. Genotypes that are not currently needed will be frozen down to reduce the number of animals bred for future needs. We will discuss proposed experimental designs with statisticians to ensure we can minimise the number of animals used while still ensuring that our data is robust. Wherever possible, we will replace mouse experiments with in vitro experiments and only use mice for validation of in vitro



validated results. All experimental results will be published in peer-reviewed journals to avoid duplication.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

A mouse's genes are around 98% similar to those of a human. It is possible to grow human tumours in immune-deficient mice to study how they behave in a whole organism, and to test out new treatments. The models developed in this study will reflect the disease with greater accuracy and will reduce the number of experiments to come to a meaningful conclusion. We will choose non-invasive methods to monitor tumour growth, which will also allow us to observe potential adverse effects earlier and end the study before the mice are showing clinical signs. Wherever possible, we will carry out monitoring and treatments at the same time to reduce stress and the number of separate interventions. When assessing a treatment in mice for the first time, we will perform a small pilot study within a range that should not cause harm, to explore its possible side effects. We will carefully choose drug doses that are effective but avoid side effects. We will plan husbandry and procedures to minimise pain and distress, observe animals daily using trained staff. We will use anaesthetic and analgesic regimes and appropriate humane methods of culling.





176. Treatment strategies for familial breast 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

No answer provided

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 is the aim of this project?

The aim of this project is to identify and test new treatment strategies for familial breast 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?

Breast cancer is the most common cancer in the UK, accounting for 15% of all new cancer cases (2016). Most breast cancer patients are treated successfully with endocrine, radiotherapy and chemotherapy. However around 20% recur. Familial breast and ovarian cancers are sensitive to certain treatments aimed at damaging DNA, these cancers also become resistant over time.

We aim to identify new treatments to kill cancers and to kill those that become resistant to current treatments.

What outputs do you think you will see at the end of this project?



Breast cancer patients are currently being treated with anti-cancer drugs that leave patients with a high risk of cancer recurrence and resistance.

We hope to provide data that will support efforts to treat these patients with agents that either prevent recurrence or that can successfully treat a tumour resistant to current modalities. Outputs of this information will be through sharing our findings at national and international conferences, through collaborations with other researchers, and by publishing in peer-reviewed journals.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The work will add to the knowledge of agents that are, and are not, effective in treating BRCA-mediated cancers (output). In the short term the outputs will accelerate scientific progress towards a greater understanding of these types of cancers (output expected during or slightly after the project).

The impact of this may be felt in the Pharmaceutical sector (those that make the new agents we test and show are successful, or make agents that also impact the targets we are inhibiting (output expected some 1-5 years after the project). Our evidence may thus accelerate trials and/or identify the patient group that the agents may be effective in (output of the current project expected to be released some 5- 20 years after the project depending on the agents used (new or already developed).

How will you maximise the outputs of your work?

To maximise the outputs we are collaborating with different groups internal and external to this establishment that will lend their expertise and knowledge to our work. We will publish our work in peer-reviewed journals and present at conferences to disseminate new knowledge including reporting of unsuccessful approaches. We will also share best practice of new techniques and protocols we have developed and validated to collaborators.

Species and numbers of animals expected to be used

Mice: 2700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Animals are injected or pieces implanted with tumour cells just under the skin on the back of the mouse or near the mammary glands. The tumours can take between two weeks up to 12 weeks to form. The tumour growth is monitored by several methods:1) measurement of a visible tumour with calipers (that provides precise measurements in millimetres); 2)feeling the abdominal area for the presence of hard lumps. With some cells, we can



monitor the tumour growth using a machine that will take live images of tumour cells that are tagged with fluorescent colour. Animals may be given anti-cancer drugs or placebo ("fake drug") by injection or by giving it orally through a long thin tube. Treatments will not exceed 8 weeks. Mice will be humanely killed at the end of the experiment. We will analyse their tumours and organs and blood to see how the drug has worked.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Mice will experience tumour growth which will not cause any pain but may cause animals to lose weight, have a less plump appearance, may sit in a hunched position, or may not act as lively as normal. Potentially, this could include weight loss (maximum of 18%), a reduction in Body condition (we use a score to monitor this), reduced activity, failure to respond to gentle stimulation, lethargic, abdominal distension, jaundice, piloerection, intermittent hunched posture, diarrhoea, or intermittent laboured respiration.

These symptoms usually appear at the later stages of the tumour development. Animals will be killed according to monitoring for adverse effects set out in the protocols.

It is possible that delivery of chemotherapeutic drugs will cause adverse effects. As the drugs we will use as well reported in the literature, we will be able to pay close attention to the development of any signs of distress. For example, anaemia and leukopenia are common side effects of chemotherapy, and we will be able to monitor the extent of this by tail vein blood 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 species)?

About 90% of animals are expected to get tumours and may also receive anti-cancer treatments and have implantation of hormone pellets/mini-pump is a surgical procedure and will be classed as moderate.

The other 10% of animals, will not get tumours or will receive low doses of agents and will have a mild severity.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We use non-animal methods to validate our treatment approaches but these conditions are artificial. They do not give an accurate picture of the effects that targeted treatment will have on the growth of tumours especially at the doses you could give to patients.



Very few, if any, in vitro cell culture based models are able to recapitulate the complex interplay between cancer cells and the tumour microenvironment. Indeed, studies using murine models of cancer that enable the modelling of disease within a complex multicellular living organism have been fundamental in enabling a better understanding of the processes that lead to malignancy that could not have been achieved otherwise. As a direct consequence of this, new drug targets and insights into the molecular mechanism of disease have come to light.

We need to use breast cancer tissue and cells in a live organism to evaluate the effectiveness of targeting a specific molecular targets on cancer development of real patient tumours.

Continued review of the scientific literature will be undertaken on a regular basis in order to identify any newly emerging technologies and models that could be potentially adopted in order to replace in vivo animal use.

What was your strategy for searching for non-animal alternatives?

In vitro data obtained from cell culture approaches has guided the proposed in vivo studies. We always conduct cell culture based experiments to justify the need to use animals. These system include culturing cells in monolayer as a homogeneous population on plastic, and the more sophisticated 3D culturing of either cells lines grown in matrigel (as acinar) or mouse/human derived tissue as organoids.

Why were they not suitable?

There are significant limitations of basic culture conditions using cells gown in isolation as a monolayer on a piece of plastic as this is not an accurate representation of what occurs within a patient. Indeed, the reason why many new drugs fail between cell culture and in vivo studies is in the inability to full recapitulate the in vivo environment. Technologies are being developed to address this gap, including the development of 3D cultures (acinar cell line cultures and organoid mouse/human derived tissue). However, none of these model systems are yet able to phenocopy the integration and interplay between the numerous cell types that constitute the tumour and its microenvironment, or the fact that tumourigenesis occur in and is influenced by biological systems (such as the immune system).

Moreover, genetic manipulation of organoid cultures is still technically challenging. Modelling cancer in mice is thus still required to fully model disease progression and identify novel therapeutic avenues.

Bioprinting and organ/human/mouse-on-a-chip are technologies that we have explored but these require significant investment in time and money to validate as they are not yet accepted as replacements for animal experiments.

The development of 3-D tissue culture of tumour cells bearing the specific genetic changes we require risks losing the precious patient material, as many of the tumour and non-tumour microenvironment cells do not survive. Nevertheless this is an avenue we are eager to continue to investigate. The advantage of in vivo propagation is it may allow us to increase the tumour material to test alternative means of 3D culture and organoid development (with REDACTED).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

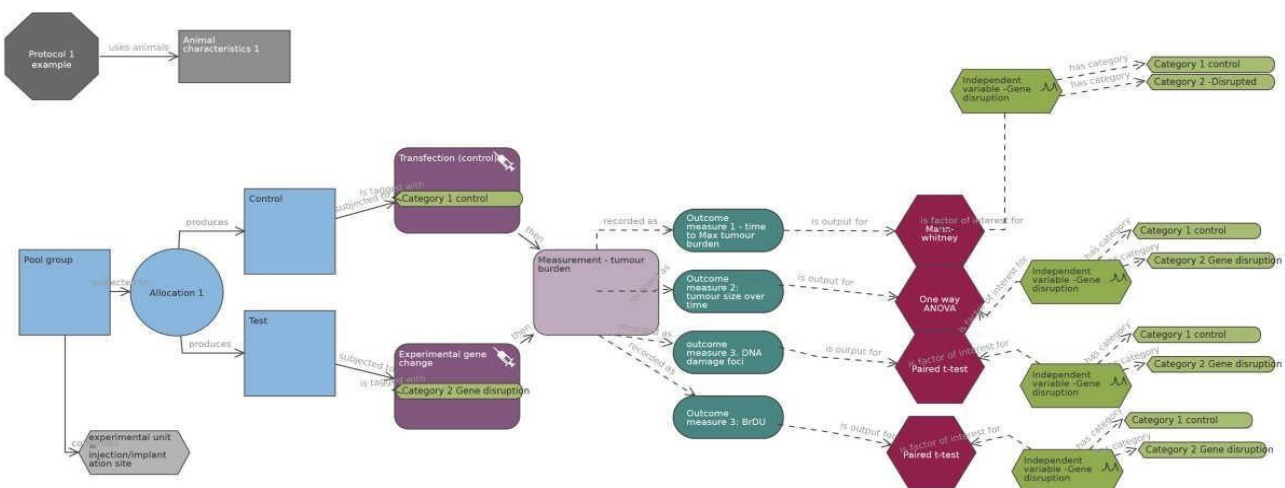
When possible we will always use power calculations to determine estimate number of animal that should be used. For most quantitative experiments, animal cohort size will be calculated via power analysis. Expected effect size will be determined through consultation of the literature, cell culture based in vitro analysis or through small pilot experiments when possible.

We have used statistical methods to calculate how many animals we need to get meaningful data, how many patient samples and how many drug combinations we will look at.

We aim to characterise about 8 patient samples each year. We aim to do about 4 treatment experiments per year. Estimating that we will test around 4 anti-cancer drugs each year.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We have used the NC3Rs' Experimental Design Assistant to design experiments (example of an experiment in protocol 1 below) and taken advice from researchers who have performed similar research projects in the past.



We aim to reduce animal numbers, methods to reduce subjective bias, and appropriate statistical analysis without compromising the scientific objectives. When possible, experiments will involve a factorial design that will maximise the information obtained from a minimal number of animals. For example, non-invasive imaging and quantification



techniques of transplantations will enable multiple measurements on the same animal over a period of time. In such cases, ANOVA will be utilised for statistical analysis.

When conducting xenograft experiments, where experimental design will allow, we routinely inject two contralateral flanks of the mouse, thereby reducing the number of animals being used by a half.

Where possible, we will use live imaging to track tumour development longitudinally. Not only does this mean fewer animals are needed overall as there is no need to cull at each time point, but it also reduces variation and so improves quality of the data produced.

What other measures apart from good experimental design will you use to minimise numbers?

We will use pilot studies to estimate variability and perform power calculations to calculate sample sizes.

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 guidelines.

We always strive to generate the most effective breeding strategies to ensure that we obtain mice of the desired genotype with minimal animal wastage. If we are unable to estimate an effect size from our in vitro data, the literature, or our collaborators, we will conduct small pilot experiments.

All tissue surplus to requirement may be deposited into SEARCHBreast (<https://searchbreast.org>), a resource to facilitate sharing of archived material derived from in vivo breast cancer 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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use mouse models to engraft human breast cancer tissue and cells. Mice with internal tumours have been refined through pilot studies where we are confident of window of treatment and experimental time point where most mice do not show any adverse effects.

We use immune compromised mice in order to achieve engraftment. These models are characterised in pilot studies to identify any unexpected adverse effects.

For human xenografts models we will use immune compromised mice, hence heterologous cells will be more easily engrafted. Imaging of engrafts will be conducted where possible through the use of non-invasive techniques that will enable us to monitor



tumour progression in living animals throughout the experiment, and will therefore reduce animal numbers. Here, the tumour volume as determined by non-invasive calliper measurements or bioluminescence imaging will be plotted against time. This design offers the advantage of determining significant differences between tumourigenic growth potential of cell lines before the limited tumour volume is reached.

In addition the pilot studies will help us to develop score sheets and robust humane endpoints so that harms can be contained within our need for a certain level of harm to answer the scientific questions.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Mice are the least sentient species that will allow us to achieve our objectives.

We need to study tumour development in adult mice as this is what most closely resemble what occurs in humans. We have chosen to use mice over other less sentient species such as Danio Rerio (zebra fish) and drosophila melanogaster (the fruitfly) as mice and humans share 97.5% of their coding DNA sequences. Moreover we are able to grow human tumour samples in these animals and assess therapeutic responses.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Through advice of colleagues performing similar work we are aware of refined treatment doses and route of administration of four of our most commonly used anti-cancer drugs so that we know the maximum tolerated dose, the minimum effective dose, and sub-optimal doses. This minimises the possibility of adverse effects from chemotherapy.

Animal welfare is a key consideration in all of our protocols and we will be guided by our NACWO and NVS in always ensuring that we are using best practice and the most refined techniques. All staff involved in animal experiments will review the literature on animal welfare provided by the local AWERB. Following every experiment and regularly during group meetings we will review our procedures from a welfare standpoint to identify any potential for refinement.

We are fortunate to have excellent colleagues both in Academic groups and within the BMSU with extensive, relevant animal procedure experience, including PDX models, from whom we can learn refined techniques from. Our team will undergo extensive training on dead animals and require to be authorised by BMSU before being allowed to perform a procedure on live mice.

Examples: Repeated injections (therapeutics) will be done on opposite sides or at different areas from previous injection so as to not aggravate any visible bruising. Gavage rather than injections for drug delivery, and vacuum blood collection rather than sequential blood sampling from tail vein. Use of refined handling where possible, depending on size and location of the tumours; cupping maybe preferable to avoid knocking the tumour (s). Humane endpoints use body condition charts.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?



We will follow the Workman et al, 2010 (reference below) for experimental design, best practice, and humane endpoints for cancer research in animals, and we will publish in journals that adhere to the ARRIVE guidelines published by the NC3R's, we consult Simon Bate's book, *The design and statistical analysis of animal experiments*, for experimental design, statistical analysis, and sample size calculations.

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 guidelines.

We will publish in journals that support the ARRIVE guidelines and conduct our experiments with advice from the PREPARE publication (PREPARE: guidelines for planning animal research and testing. Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. *Lab Anim.* 2018 Apr;52(2):135-141. doi: 10.1177/0023677217724823. Epub 2017 Aug 3. PMID: 28771074).

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How will you ensure you continue to use the most refined methods during the lifetime of this project?

Literature searches, attendance at vendor's information sessions, seminars and conferences to find out about new technology and new approaches that we could implement.

We will comply with the ARRIVE guidelines (*Animal Research: Reporting In Vivo Experiments*; www.nc3rs.org.uk/arrive), a NC3Rs-developed checklist of the essential information that should be included in publications reporting animal research.

My Lab group twitter follows the NC3Rs - helping us keep up with advances in the 3Rs which will be discussed and implemented through our lab meetings.

Explain the choice of species and the related life stages

We are using mice because they are able to grow human tumour material as xenografts enabling drug testing and in vitro genetic manipulation before implantation. This enables us to mechanistically interrogate tumour growth, and hence identify new way in which to treat breast cancers.

A number of our approaches require the growth of human cancer cell lines or tumour pieces, and this has to be conducted in mice that have a disabled immune system to



prevent human cell rejection. We choose to use adult mice of a specific age range (6-9 weeks of age) to minimise variability.



177. Tumour Growth Regulation

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.

Key words

cancer, sarcoma, therapy, REDACTED

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

The work aims to understand how cancers grow, so that we can develop ways of blocking cancer growth in man. This understanding will direct our potential to develop new drugs for treatment of childhood and adult cancers. Based on a thorough understanding of molecular mechanisms, this work will not only inform what drug therapy, but also how to best apply that therapy so that it is administered to the right patients at the right time. The specific objectives include determining the activity of one such drug therapy, that acts to block cancer growth. The effects of REDACTED treatment on cancer growth will therefore be assessed. In addition, we will determine what genes promote growth in rare cancers called sarcomas, so that we can develop new treatments. Sarcomas are cancers with unmet need. They affect children and young adults, and despite treatment with mutilating surgery, high dose chemotherapy and radiotherapy, and still associated with poor survival.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

The benefits are directed ultimately towards developing new treatments for cancer. Initially, the benefits will be to generate new knowledge, to identify new cancer targets and to validate these, to then develop evidence of new agents that modify these targets as the basis for new treatments. For the clinical trials that then follow, the benefits are for humans



that might have cancers appropriate for treatment with the new drugs, and for society following commercialisation of new drugs that show benefit for cancer patients.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Species: Mouse

Number: 1050

Time: 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

In the protocols, adverse effects relate to the induction of growth of tumours placed under the skin, and following injection of agents and tracers. Both may impact on the animals, but the likely level of severity being moderate.

Continuous monitoring of symptoms and signs will be performed so as to minimise impact of procedures and to avoid rare adverse effects (<5%), including limiting the size to which tumour will be allowed to grow. The growth of tumours will therefore be monitored with ultrasound imaging, but in addition, animals will be treated with therapeutic substances to try and prevent tumour growth. Animals will undergo anaesthesia prior to procedures in order to minimise adverse effects such as pain. All animals will be humanely killed at the end of all the protocols.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

There are currently no alternatives to animal models when the objective is to study the genetic and functional effects of a growing tumour arising in a whole animal context that aims to mimic the situation in human cancer. We have replaced stages of some experiments, for example, in the early stages of designing an experiment, we now routinely use new CRISPR/Cas9 genome editing technology in cells in culture, as this optimises genetic controls in cancer models that are more informative and that can replace the use of genetic modification in animals. Alternatives to animals are also being developed in several laboratories, and are based on growth of cells in small chambers (cancers on a chip), and may be also utilised as an alternative by us in the future.

Reduction

Explain how you will assure the use of minimum numbers of animals.



To reduce animal numbers, we have adopted a number of approaches. Firstly, we will use optimal genetic controls to compare against the test substance. This approach reduces the numbers of animals needed, as controls are specifically designed. These controls can be tested in cell culture screens, and as a result, optimises the relevant combinations of treatments requiring testing, and so significantly reduces the number of animals required. Secondly, we will image tumour growth in experimental and control groups using accurate non-invasive ultrasound, so reducing experimental variation due to measurement error, requiring less animals to meet the need for statistical power. We will also use standard experimental design that incorporates blinding, randomisation and gender differences.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

We will utilise humane endpoints and imaging throughout the protocols to quantify early stages of tumour growth minimising the risk of mice developing potential symptoms from tumours that reach the limit of size. By objective and regular evaluation with ultrasound monitoring, we will be able to intervene earlier. We also routinely ensure that all animals receive anaesthesia to avoid pain, we will utilise LASA guidelines, and if animals exceed a predetermined humane endpoint before suffering or harm, to then be killed humanely.





178. Type 2 immunity in infection and maintenance of tissue health

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo
Gerbils	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 is the aim of this project?

We want to understand how the immune system functions to protect the host from parasitic worms, which cause damage as they migrate through the body. By extension, we aim to learn how these immune pathways help heal wounds or cause disease by overzealous tissue 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?

Helminths (parasitic worms) infect over a quarter of the human population, and an even greater proportion of animals. Although not generally fatal, they cause substantial suffering, particularly in low income regions. Disease can be caused by competition for nutrients, physical injury due to migration through the body, and/or an excessive immune response to the parasite. The type 2 arm of the immune system is a highly complex network of cells and molecules, which is very distinct from the pathways that control



infection with bacteria and other microbes. Type 2 immunity is necessary both to control helminth numbers and to repair the physical damage they do. However, overzealous type 2 responses can lead to allergic diseases, asthma and chronic tissue scarring (fibrosis). Wound repair and scarring features of type 2 immunity are common even in regions with no helminth infections. Thus, fundamental research into the mechanisms and consequences of the type 2 immune response will lead not only to improved understanding and control of helminth infection, but greater understanding of the many allergic and fibrotic diseases that are major killers worldwide.

What outputs do you think you will see at the end of this project?

This project aims to disseminate new knowledge by publication in peer reviewed journals and presentations at conferences, seminars and workshops. We hope that in the longer-term our work will contribute to new immunology-based therapies. In the 5 years, we aim to continue our high standard of publication, averaging more than 5 research papers per year in highly-respected peer-reviewed journals.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

This project aims to answer fundamental scientific questions. The new knowledge generated, and the unravelling of important immune mechanisms, will be relevant to a broad range of human and animal conditions.

Parasitic worms (helminths) afflict over 1/4 of the human population and the vast majority of wild mammals. They also represent an enormous economic burden in terms of livestock productivity. In the next five years we expect to make significant strides in understanding the mechanisms by which these parasites are killed, which will be relevant in the longer-term to prevention or treatment strategies. In addition, helminth infection can increase susceptibility to other infectious agents, such as viruses and bacteria. Our analysis of how cells function during helminth infection should help us understand how to manage these common co-infections.

Our work is directly relevant to fibrosis (tissue scarring) and asthma. Fibrosis in people is estimated to contribute to 45% of all deaths in developed countries. In the UK, around 5.4 million people are currently receiving treatment for asthma, which equals 1 in every 12 adults and 1 in every 11 children. We expect our work over the next 5 years to define the key molecules involved in how the tissue becomes restructured and remodelled to cause more severe disease, and potentially whether the process can be reversed once it has started. This is particularly relevant as new biological drugs that target type 2 immune pathways are now used in the clinic for skin disorders, and our studies would highlight their potential role in other diseases.

Because several of our models involve the investigation of the body cavities, they may provide important insight into a major clinical problem: the adhesions (inner body scars) that follow abdominal surgery. The underlying cause of these very painful adhesions are poorly understood, but have been linked to the immune cells we study. Our unique expertise in the cavity around the lung (the pleural cavity) will also provide insight into build-up of fluid in the cavity resulting from heart failure, pneumonia and cancer. In particular, we are interested in the possibility that idiopathic pulmonary fibrosis, a fatal lung disease of unknown origin, may start in the pleural cavity. Our work with a parasite that



lives in the pleural cavity, has the potential to reveal whether scar tissue initiated in the pleural cavity can move into the lung.

One of the most specific outcomes we hope to achieve is an understanding of the function of chitinase-like proteins. Increased levels of these proteins in the blood are markers of poor outcome in a wide variety of diseases from asthma to cancer but their function is unknown. We hope to reveal why chitinase-like proteins are associated with disease and whether drugs designed to block them could be beneficial. This work could lead to new therapeutics in the next 10 to 15 years.

How will you maximise the outputs of your work?

Communication of our findings will be primarily through publication in widely-read peer-reviewed journals, but also presentation at local, national and international congresses and institute seminars. To ensure maximum dissemination, only journals that allow open access without payment by the reader will be considered. Furthermore, we will place the first drafts of our published data on an open access repository such as www.biorxiv.org. To prevent unnecessary repetition of experiments by others, we will seek to publish all data generated under this project including negative results.

To enable rapid translation of our findings to the clinic we will exploit new and existing collaborations with local clinicians as part of the translational environment within our institution. We have highly effective systems in place for technology transfer. Additionally, pharmaceutical and biotech companies could be engaged through presentation at national and international forums at which representatives are often present.

Species and numbers of animals expected to be used

- Gerbils: 500
- Mice: 40000
- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically a mouse will be either 1) infected with a parasitic worm by injection under the skin, 2) injected with a molecule that induces a scarring response, or 3) given a mixture of allergy inducing molecules through the nose. Experiments might look at the immediate immune response in the first few days but can last as long as 3 months to allow full development of the parasite, or full development of diseased tissue (e.g. asthmatic lung).

Many animals will also receive an injection of a molecule or cells to modify the immune system. For example, an animal might receive an antibody that will get rid of a particular immune cell. In addition, small volumes of blood may be taken from a vein, for example to screen for parasites or blood cell changes. Experiments will often end with animals being killed under terminal anaesthesia.



Hence, the cumulative experience of mice will typically be exposure to 2 or 3 procedures that may each cause short but usually separated periods of typically mild or potentially moderate degrees of suffering.

Separate from the above experiments, some genetically altered animals will be used only to breed and maintain animal lines.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The vast majority of animals will experience no adverse effects or only mild adverse effects. The parasite infections are generally well tolerated and will rarely reach moderate severity. In the hookworm model a heavy infection can cause weight loss and laboured breathing but we use low doses to avoid this. The fibrosis and allergy models are designed to assess the structural changes to the tissues without major overt clinical symptoms. However, some manipulations can make animals more susceptible to infection, injury or allergy, which may increase the severity from mild to moderate. All animals will be humanely killed before they exceed moderate severity limits.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Based on our previous experience, we expect approximately 85% of mice to experience mild severity and less than 4% to experience moderate severity. The gerbils and rats all experience only mild severity.

What will happen to the animals at the end of the study?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune system relies upon a complex series of interactions, which occur between immune cells, structural cells and the extracellular matrix. The nematode models involve migration of parasites through living tissue. To understand such complexity, it is essential to undertake research in vivo, since this cannot yet be modelled meaningfully in vitro.

What was your strategy for searching for non-animal alternatives?

Analysis of human biopsies.

Use of cell lines



Use of organoids - artificially grown cells that resemble an organ.

Why were they not suitable?

The types of experiments required to track cell function in vivo are not possible with human tissue biopsies, nor can we experimentally infect humans.

Many location-specific features of cells are lost once they are removed from the tissue, which makes the use of cell lines impractical.

Organoids cannot test the impact of nematode migration through the body or replicate the changes in the extracellular matrix and cell migration that occur during injury, tissue remodelling or scarring.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 Rats and Gerbils, the calculation is based on our current requirements for maintenance of parasites. The number of mice has been estimated based on experience gained under my previous Home Office licenses. This experience-based estimation has reduced the predicted animal use by over 30% compared with my previous license.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Maintenance of parasites: On the previous license we estimated 8000 animals for lifecycle maintenance - our procedures have improved dramatically such that we have reduced our estimate for this license to 5050. For mite feeding, we have reduced both the number of mice and the number of infected gerbils needed.

Experimental mice: For all of our experiments in-bred mice are used to reduce experimental variation, which makes it possible to use fewer numbers of animals to achieve statistical significance. For the majority of our studies, mice from the same litters are used for control and experimental mice, reducing variation that can occur due to differences in the microbiota. Overall, our experiments are designed to reduce the number of variables (for example age) to as few as possible and thereby reduce the number of control groups required.

We work with the NC3Rs Regional Programme Manager to ensure all lab are introduced to NC3Rs experimental design assistant and encouraged to use it. Everyone in the lab is trained in statistical methods and these are regularly discussed at lab meeting, to ensure all agree the best methods are being used. Tissue-sharing is a major tool we use to reduce animal usage.

Breeding: A significant proportion of our animal use is related to breeding programmes for GA lines. We follow the advice of our BSF staff to optimise breeding and regularly discuss



numbers at lab meeting to ensure we do not overbreed. Our breeding number estimates have been considerably reduced from my previous license. Where possible and appropriate, we use antibody blockade instead of gene knockout mice.

What other measures apart from good experimental design will you use to minimise numbers?

My group routinely perform pilot experiments to determine the optimal number of mice to achieve statistical power. Experiments are then performed on a minimum of two separate occasions to ensure reproducibility, following which data pooled from experiments are statistically analysed to reveal less pronounced effects without increasing overall animal use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use mouse models of nematode infection, fibrosis and tissue remodelling. Mice represent the most appropriate species for in vivo study of these conditions, because of the extensive knowledge of their physiology as it relates to humans, the genetic and biological tools available and the ability to be easily bred and handled.

The nematode infection models do not cause significant pathology and we use them to understand the immune response to the infection process. The infectious doses are carefully managed such that the animals will experience minimal suffering.

Our model of fibrosis will result in scar tissue and has been chosen to mimic what happens to people who undergo peritoneal dialysis. The model is terminated at a point where the causes of scarring can be assessed but before the animals experience evident suffering. Our model of severe asthma has been designed to generate remodelling of the lung airways and study the causes of airway stiffening that lead to asthma. The models are designed to allow us to quantitatively assess substantial remodelling of the lung airways, in order to mimic human disease. However, the experiments are terminated before the animals exhibit serious breathing problems.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We are studying long complex processes and trying to understand how the adult immune system handles these conditions and how different tissues communicate to orchestrate an appropriate response. Only adult animals would give meaningful results. The long duration of the processes to be studied prevent using mice under terminal anaesthetic.



What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

In line with the establishment's policy, we will adopt the latest techniques in animal handling (eg cupping) to significantly reduce the stress associated with procedures. Furthermore, where possible, the least invasive methods for dosing and sampling will be applied.

Anaesthesia and analgesia will be provided where suitable (eg for humane restraint, during and recovery from surgery). The best aseptic technique will be used during surgery.

Infection experiments will use the lowest doses possible and early endpoints will be used that prevent animals experiencing more severe harms.

We are continually refining our procedures with regard to the *Litomosoides*/mite lifecycle and over the years have made dramatic improvements for which we have won 3Rs awards. We are acutely aware that the gerbils are social animals and work to avoid isolation where possible. We recently moved to refine blood taking from the gerbils by switching from tail vein bleeding to saphenous vein bleeding, a procedure that is less stressful for the animal and easier to quickly get sufficient blood. Another refinement is that the gerbils are given treats after being handled and now exhibit minimal anxiety on being handled. Best practice is discussed regularly at lab meetings. In addition, the lab manager has been sent to collaborators labs to discuss and observe their practices and see if our practices can be refined. This has resulted in continual improvements to our life cycle processes.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The lab consults the recommended <https://www.nc3rs.org.uk/3rs-resources> on a regular basis including watching videos of best practice techniques. For specific models, we read papers from other groups doing similar experiments, as well as consulting directly with other researchers to discuss the most refined procedures.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The animal facilities includes a team of dedicated veterinarians that are continually seeking to improve animal welfare and refine animal use. My group consult closely with them and take full advantage of the extensive resources provided to ensure we are following current best practices. We are also in the process of adopting the improved rodent handling methods that reduce animal stress (detailed by Hurst et al. Nat Methods 2010) and now provide environment enrichment as standard. We will regularly consult with the NC3Rs representative and their resources page to ensure we are aware of new 3Rs developments.

Explain the choice of species and the related life stages

We study adult mice because the immune system, tissue organisation and development of all mammals are similar allowing mice to be a model for humans and other animals. We also use mice because scientists have created many genetically altered REDACTED that allow us to dissect in fine detail what happens during immune responses. Genetically altered mice and many of the tools designed to work with mice allow us to define in precise



detail how particular cells and particular molecules work together to repair tissue or fight infection. By manipulating these cells and molecules, we can understand how these processes go wrong during diseases such as asthma.

We also use gerbils and rats to maintain our parasite lifecycles, and neonatal mice to maintain the mite vector that transmit parasites. In each case, the animal is the most susceptible to infection, allowing us to use the least numbers of animals to maintain the parasites or mites in sufficient numbers for experiments.





179. Understanding colorectal cancer risk factors and progression of disease

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.

Key words

No answer provided

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

This project aims to identify and characterise CRC risk factors and understand how different parameters (genetics, age, sex, environmental, diet, tumour promoting/preventing factors) impact on pathways involved, to enable appropriate and personalised intervention and treatment.

The objectives are:

To understand how CRC risk factors contribute to normal cellular function, tumour initiation, development and progression in order to be able to intervene.

To understand what and how environmental and genetic parameters (eg. genes/pathways) contribute to tumour initiation, development and progression.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

CRC has a high death rate, however, if detected early, this rate decreases dramatically. Understanding the risk factors associated with CRC can help tailor screening programmes



to those most at risk and we can better understand pathways to target therapeutically and indeed intervene with pre-clinical trials of potential anti-cancer therapies/preventions.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Up to 10,000 mice will be used over 5 years.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The programme of work will be -

To alter CRC risk genes to understand their contribution to tumour development and progression as well as their normal function.

To create combinations of mutated genes in order to determine which gene mutations contribute to tumour development and what parameters and factors affect this and how.

To use or create exposure to potentially tumour causing and tumour preventing agents to understand how environment may contribute to or modulate tumour development.

Due to the nature of the experiments to understand tumour formation, progression and prevention, adverse effects are expected as a result of aging; tumour development via genetic background, tumour promoting agents and inflammation; dietary manipulation; injections; multiple anaesthetics; surgery; imaging; single-housing. The animals will be housed in a modern animal care facility and monitored daily for signs of illness (using a number of Veterinary agreed monitoring and scoring systems including weight loss, distress and clinical factors) due to tumour formation and for other well being factors. The expected adverse effects due to tumours will be kept to a minimum (mild/moderate level of severity). At the end of the studies, mice will be humanely culled and dissected to analyse various tissues for tumour formation/progression.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Cancer formation is complex and study in mouse models will allow us to fully understand pathways and different factors linked with tumour formation.

We will utilise mice to investigate CRC risk factors, as they are similar to humans in terms of numerous features (anatomical, genetic, pathological and physiological). We have shown by analysing human intestinal tissue, that CRC risk factors have a larger effect in the tissue of disease origin and therefore we can use genetically altered mice to analyse particular tissues and investigate stages of tumour development, including any interactions



between genes, cells, tissues, the surrounding microenvironment, with and without the impact of other factors.

Functional studies involving ex vivo samples (growing intestinal crypts, tumours, human cancers) and in vitro (cancer cell lines) will be performed before and during the studies, however understanding the complexity of tumour formation in its surrounding tissue structure is critical and these mouse studies will recapitulate the human disease.

Throughout the project we will seek, review and incorporate alternatives as appropriate.

Reduction

Explain how you will assure the use of minimum numbers of animals.

The experimental design has been optimized to ensure use of minimal animal numbers to produce meaningful results and is helped by our previous projects.

We plan to use efficient breeding practices/sourcing methodology and utilise imaging technologies to help to reduce animals where possible.

We plan to use ex vivo intestinal crypt culture studies and in vitro cell line pilot studies for functional analyses of gene effects.

We will harvest as many of the tissues possible, for archiving and storage, to minimise repetition of experiments.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Mice are chosen in this research due to their small size, relatively short lifespan, rapid reproductive cycles and similarity to humans in terms of many different features. Mice are prone to both spontaneous and induced tumour formation. As they are more defined genetically, they allow a much greater insight into the roles of specific genes, alterations and environmental effects. We will use existing mutant mice where possible and indeed utilise the intestinal crypt culture to both reduce and refine the research.

We will use anaesthesia and analgesia as appropriate, with guidance from NVS and utilise imaging technologies to help refine the procedures.

The animals will be housed in a modern animal care facility with barrier housing to minimise infection and harm. The staff are well trained and we will ensure they receive the highest standard of care with appropriate stimulation to allow social enrichment where possible. The animals will be assessed daily and we have developed a scoring system with guidance from the NVS, to ensure that animal suffering is kept to a minimum, with animals being culled when mild-moderate clinical signs are observed.





180. Understanding haematopoietic dynamics in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

No answer provided

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 is the aim of this project?

We aim to understand how the cells responsible to sustain blood production (blood stem cells) are affected by stresses such as infections or leukaemia growth. Because it is not yet possible to maintain and grow blood stem cells in a test tube, we study them directly in the bone marrow, where they reside, with the aim to understand what other cell types interact with them, exchanging what molecular signals.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite bone marrow transplantation having been practiced in the clinic for decades, and being the treatment of choice for an increasing number of conditions, availability of blood



stem cells is a critical limiting factor for its applicability. Understanding what cells and molecular pathways support blood stem cells in the bone marrow is critical to learn how to grow them in test tube so that more patients can be treated successfully. Moreover, it is now clear that blood stem cells are damaged by stresses such as infections and leukaemia, therefore it is important that we learn how to preserve them or regenerate themselves following stress. This will enable healthy ageing.

What outputs do you think you will see at the end of this project?

By the end of this project we will have significantly increased the understanding of blood stem cells and leukaemia biology, and identified specific cells and molecular pathways that are promising for the development of novel, improved therapeutic approaches for leukaemia and cancer patients, and for survivors of severe infections. Our findings will be published in highly respected journals (always in open access format) and presented at prestigious conferences. All data generated will be accessible to the research community either directly through deposition in existing databases or upon request by collaborators.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Our data will be useful to drive future research led by us, other research groups and industry in collaboration with clinicians and to drive the development of improved therapeutic and preventative approaches for blood diseases. Patients will be the ultimate beneficiaries of our work.

Outputs from our work will immediately benefit the closer research community, and will reach the global research community once they are published. In the medium term (2-10 years) they will underpin future research projects led by me and others, including potentially clinical trials that test novel therapeutic approaches. In the longer term (5-20 years), our findings will impact human kind in terms of improved therapeutic and preventative approaches in the areas of leukaemia, cancer and infection, and will therefore contribute to develop strategies to enable healthy ageing.

How will you maximise the outputs of your work?

I strive to publish all our findings, positive and negative. This is becoming more feasible through the increasing availability of open access data repositories and pre-print publications (e.g. Wellcome Open Research, BiorXiv). Moreover, both myself and trainees in my group regularly present our findings at scientific meetings, and engage with the public and, most importantly, with the technology development, technology transfer and intellectual property departments of both the institutions and the funders who support us. This maximises the opportunities for our work to be evaluated and further developed to drive improved therapeutic and preventative approaches.

Species and numbers of animals expected to be used

- Mice: 16,250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.



Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

The length of our experiments, both in terms of actual time they take to be completed and of number of procedures performed on an animal, varies greatly depending on the questions asked and the most relevant models used. For example, our infection models develop within 5-7 days, but our leukaemia models develop within 3-12 weeks. Infections are mimicked by injecting specific substances intravenously or intraperitoneally, or by administering malaria parasites intravenously or via the bites of infected mosquitoes. Leukaemia grows following intravenous injection of malignant cells, with or without preparing the mouse with agents such as irradiation. In the vast majority of cases mice will develop either infection or leukaemia, and not both. Only in later stages of this project, once we have learned the specific damages caused by infection, we will let the mice fully recover from infection and we will subsequently administer leukaemia cells to them, asking whether they are now more susceptible to develop leukaemia. Within these models, we treat mice with substances that activate genes or modify cells that regulate blood stem cell function. These could be hormones administered over 2-4 weeks, or chemotherapy leukaemia treatment administered for 5-10 days. To assess the functionality of blood stem cells, donor mice will be culled and blood stem cells harvested and transplanted into irradiated recipients, mimicking bone marrow/stem cell transplants performed in the clinic. Specific to our programme of work, we will use advanced microscopy to directly observe blood stem cells and support cells interacting within the bone marrow. This is done under anaesthesia, either as a terminal or recovery procedure. Less frequently, longitudinal studies will require multiple, short microscopy sessions to take place over the course of a few days.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Mice will experience either mild or moderate levels of severity. Moderate levels of severity are expected for all mice undergoing recovery surgery, and for those experiencing multiple mild procedures within a short period of time (for example daily blood sampling or daily injections).

We expect adverse effects to be very rare (<2% of animals). An exhaustive body of evidence demonstrates that stress affects HSC biology, and it is therefore paramount to us that stress, such as that generated by adverse effects, is minimised, or else we would not generate trustworthy data.

Peri-operative analgesia is provided and animals are expected to be fully recovered within 24 hours, most often within 2 hours from the end of surgery/anaesthesia. Very few animals will undergo more than one surgery (for example, two bone marrow aspirates, or one bone marrow aspirate and one imaging window implantation), and the second surgery will only be performed once full recovery from the first one has taken place and it is clear that that did not cause any adverse effects. We are not currently planning to perform any further surgery on splenectomised mice, and should this become necessary we will discuss it and plan it in tight collaboration with NVS and NACWO, and following the same principle of performing a surgery only if the animal has not shown any adverse effects at any point.



Irradiation is not expected to cause any adverse effects to our animals because either they will be culled before these arise or they will receive sufficient numbers of haematopoietic cells to guarantee their survival. In the latter case, mice will be cytopenic for 3-4 weeks, with the nadir at week 3 post- irradiation. While this is an adverse effect, this does not cause observable clinical signs to the mice. The worst-case scenario is failure of the injected cells to reconstitute the mouse, however this is never planned and therefore it is not an expected adverse effect for this project. If further procedures need to take place following irradiation and injection of cells, these take place either before or after the time window when mice could develop adverse effects, or are terminal procedures performed under anaesthesia (eg intravital microscopy).

Leukaemia cells can cause health deterioration which is only ameliorated by chemotherapy treatment. Animals are expected to respond quickly to treatment and clinical signs to resolve within 24 hours, otherwise humane killing is triggered. Malaria can develop into cerebral complications, which are lethal. Our experiments are all performed before the onset of these complications, and the onset of signs such as reduced motility and hunched posture is carefully monitored (daily inspections) and triggers humane killing.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Expected moderate: about 30% of animals

Expected mild: about 70% of animals

What will happen to the animals at the end of the study?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is currently impossible in the laboratory to mimic the complex three-dimensional relationship between normal blood stem cells and other cells in the bone marrow, and therefore completely replace the use of animals.

Mice are the smallest, less-sentient animal model that can be used to perform the studies described here. They are the only species for which there is a wide availability of genetically altered animals, which makes it possible for us to test the role of specific genes within the blood production system and to visualise cells of interest directly within the bone marrow. Finally, the blood production system that we are studying is remarkably similar to the human system.

What was your strategy for searching for non-animal alternatives?



We continuously strive to replace animals as much as possible, and provide here some examples. We have implemented mathematical modelling to test theories about certain aspects of blood stem cell biology and simulate interventions. This work allows a partial replacement of the use of animals as it allows us to select parameters to be tested experimentally and therefore reduces the number of animals used. We have also been using our findings from animal studies to develop laboratory systems. For example, we reproduced the disease effects of leukaemia on the cells normally supporting blood stem cells by growing both types of cell in the same experimental dish, and we have been studying the interaction with other cells using similar approaches. In many cases these systems rely on cells taken from mice to set them up, but they do allow us to perform further studies without the need to use more animals although it is only made possible in conjunction with our research findings in mice.

Why were they not suitable?

Commercially obtained cells or those grown in the laboratory are not suitable replacements for cells freshly taken from mice because they are too different and any experimental results would not be relevant to our research.

Specialised techniques using cells are increasingly used in our type of research, but are not yet suitable for research involving blood stem cells, however our findings from animal studies will contribute to improving 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 use statistical modelling to estimate the ideal number of mice we need in order to achieve meaningful scientific results.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

When statistical modelling indicates that numbers needed are high (for example more than 15 or 20 mice), we use preliminary pilot studies with smaller numbers of mice. Depending on the results, we either abandon that experiment if it is shown not to work or adjust, and often reduce, the number of animals needed if the pilot results are promising.

What other measures apart from good experimental design will you use to minimise numbers?

We breed mice that can have more than one particular trait which minimises the number of animals that we need to use.

By using the same control (untreated) animals and by increasing the breadth of analyses conducted on cells and tissues from each animal, which gives us maximum data per



animal, we have in the past been able to use smaller numbers of animals than initially expected. We expect this trend in reduction of numbers to continue in future.

Careful planning of microscopy allows us to follow events as they develop within the same mouse, avoiding issues due to mouse-to-mouse variation and therefore reducing the total number of animals that need to undergo the procedure. Moreover, careful refinement of mouse monitoring and surgery/anaesthesia reduces animal death and ultimately the number of mice required to complete data collection.

In addition, we maximise our breeding efficiency by minimising the number of pairs required through careful crosses and monitoring of all breeding animals.

Allowing malaria parasites through their whole life cycle including in both mice and mosquitoes maintains parasites stocks more consistently, reducing variability and the number of mice required to complete data collection.

The use of state-of-the-art hormone administration protocols increases the efficiency of embryo production, reducing the number of females needed to generate the embryos required.

Finally, as multiple personal licence holders work under this licence, experiments are coordinated so that tissues can be shared as much as possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The experimental models of leukaemia and infection used in this project have been selected to minimise pain, suffering and distress to the animals, while providing scientifically relevant data. No lasting harm is experienced by any of our animals.

Of note, the use of naturally occurring infectious agents is a refinement as it eliminates the question whether the observations made following administration of large amounts of specific molecules are relevant to real-life scenarios. Still regarding the infection models we use, and in particular malaria infection, opting for infected blood inoculation rather than insect bites whenever compatible with the specific scientific question addressed reduces the suffering of animals as it does not require anaesthesia and it shortens the overall duration of the infection.

The use of sophisticated genetically altered animals where mutations are restricted to specific cells of interest instead of affecting the whole organism drastically reduces the emergence of adverse effects due to the genetic background of the animals. For this reason, our breeding protocol leads to only mild levels of severity experienced by the animals.



The use of sterile males whenever possible reduces the need for vasectomy and therefore avoids using surgery to achieve the same output.

Finally, throughout our experiments, mice are carefully monitored at a minimum through daily inspections, which increases to twice daily at times when adverse effects are expected (for example at late stages of leukaemia development, or the peak of malaria infection). Monitoring may be undertaken continuously in some situations, for example for the few hours following recovery surgery, to ensure recovery is smooth and prompt.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The mouse is the ideal model organism to study blood stem cell systems as it is most similar to the human. Less sentient animals such as zebrafish and the fruit fly can be used to ask very specific questions, but their blood stem cell system is too different from the human to address our research questions.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Our past experience has brought to our attention specific adverse effects that can be monitored and minimised. For example, we refined our breeding protocol (P1) to identify certain genetically altered animals by eye inspection using fluorescent goggles whenever possible. As a result, we have been able to eliminate tail clipping as a procedure.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

LASA guidelines for the administration of substances are followed at all times. Moreover, NACWOs and NVSs bring to our attention relevant publications describing practices that we can implement. For example, as a result of such interactions we have implemented the body conditioning score system to ease the identification of human end points in all our experiments.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

Because this is a follow-on project licence (PPL), the majority of the protocols have been revised and refined from the REDACTED I held.

All protocols undergo constant refinement as new and improved approaches are developed that minimise mouse discomfort and allow better welfare monitoring. Continuous collaboration with local Named Animal Care and Welfare Officers (NACWOs) and NVS ensures that our work is constantly refined and always up to date with any 3Rs advancements. In particular scheduled PIL re-trainings have been proving an excellent approach to ensure implementation of any 3Rs advances.

Explain the choice of species and the related life stages

We work with mice because their blood production system is remarkably similar to the human one. Moreover, they are small, and can be genetically altered, allowing us to test several theories about the cells and molecules causing damage to blood stem cells during



leukaemia and infection stress. We work with young to mature animals, typically between 4 weeks and 6 months of age at the beginning of our experiments. This is because we model human adult blood production, and in the mouse, it has been shown that by three weeks of age blood production has completely switched from the foetal/developmental type to the adult/steady-state one.



181. Understanding immune modulatory processes to prevent and treat pathology and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

No answer provided

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 is the aim of this project?

The immune system fights off infections and ensures the well-being of the body, but is also the cause of a wide range of different diseases, such as allergies, cancers or chronic fibrotic diseases. It is the aim of this project to understand how the immune system is balanced so that it governs the well-being of the body. Based on this knowledge we want to develop novel therapeutic approaches to prevent the development of pathology 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?

The immune system co-evolved with mammals. It protects the body against infections and regulates a wide range of processes of the body. For instance, during infections, immune responses ensure that pathogens can efficiently be cleared. At the same time, the immune response also initiates a wound healing process so that tissue damage can be kept to a



minimum. Failure to regulate these processes in the correct way are an underlying reason contributing to the development of inflammatory chronic diseases, such as auto-immune or chronic fibrotic diseases, as well as cancers. In order for us to be able to find new therapeutic approaches to treat and heal these diseases, it is important to understand how they develop and at which stage the immune response has become dysregulated. This project will address these different processes.

What outputs do you think you will see at the end of this project?

The experiments performed under this licence will give a deeper understanding of a physiological relevant mechanism which is mediating local immune modulation.

We expect that this knowledge will then help us to understand how these mechanisms shape immune responses so that they can efficiently clear pathogens but do not cause autoimmunity. In line with this understanding, we may also better appreciate how commonly used immune modulatory drugs, such as TNF α inhibitors, actually work. Such results may then allow us to develop alternative immune modulatory drugs that may complement or replace currently used drugs.

Furthermore, we expect that this knowledge will reveal how immune responses maintain tissue homeostasis under inflammatory conditions and thereby prevent the development of pathology, such as tissue fibrosis. In the context of this licence, it is the aim to further develop and test novel drugs to target the pathological processes, in order to prevent further disease progression and to revert pathology.

Finally, experiments performed under this licence will reveal whether tumours are using this immune modulatory mechanism to establish a tumour-intrinsic immune-suppressive microenvironment and whether we can use specific therapeutic approaches to disrupt this immune-suppressive microenvironment and to enable immune mediated rejection of tumours.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Each of these three different topics affected by experiments performed under this licence are areas of great research activity. Thus, any results from our experiments will be of great benefit for other researchers as well as for clinicians working in this field.

Several experiments will also address the underlying mechanisms of drugs already approved for clinical application. Thus, our experiments may suggest for a better educated application of these drugs or the application of these drugs for other/novel disease descriptions. Thus, a wide range of patients suffering from auto-immune or chronic inflammatory diseases or cancers could directly profit from our research.

Finally, all these different areas of research have the clear potential for the development of novel therapeutic drugs, that could complement or replace drugs already approved for clinical application. Following further development of these drugs for clinical application and clinical trials, these drugs could then potentially further enable the treatment of patient groups - potentially also of patients suffering of diseases, which currently cannot be treated.

How will you maximise the outputs of your work?



A critical aspect of maximising the effect of our research is to find recognition by key, influential people in the field. These could be other researchers or clinician or stakeholders in companies. To achieve this, we will aim of publishing our results in recognised journals and to "spread the word" in social media. Furthermore, it is important to attend influential conferences in the field and to present our work.

Furthermore, critical for the translation of our findings into clinical approved drugs is to find collaborations with pharmaceutical companies, with whom together we will develop our research findings into translation-able compounds.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Using well-established, standardised methods, animals will be immunised, given an infection or auto- immune diseases will be induced (Objective 1).

Wounding can actively be introduced or chronic inflammation induced. Similar to human asthma or atherosclerosis, also in mice such chronic inflammation will then lead to pathology (Objective 2).

Tumour development will be induced (Objective 3). This can be slowly on a predisposed genetic background, or this can be done by the transfer of tumour cells. Tumour growth will then be blocked using different immune mediated approaches .

To experimentally manipulate the immune responses in above mentioned model systems, mice may receive substances or cells via injections. Also their food and drink might be altered (high-fat food to induce atherosclerosis) and animals may inhale allergens.

The infection models are relatively short (about one week), the auto-immune models last longer (about 6 weeks), while the tumour and chronic tissue remodelling experiments tend to last over several months.

All animals are closely controlled during the experiments and euthanised at the end of experiments.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Almost all experiments have minimal impact on the well-being of the animals.



Similarly as to what is known for humans, also our animals may not yet experiencing any discomfort although, for instance, their atherosclerosis might have progressed substantially, or their asthmatic lungs might already have lost a substantial fraction of their lung capacity, or a tumours might have grown to a substantial size. These are all critical read-out measures for our experiments, but at this stage may not have caused any discomfort to animals, yet.

Nevertheless, some infection models may lead to transient weight loss (still moderate) and some auto- immune models may for instance lead to substantial, transient paralysis, as seen for the mice undergoing EAE, the mouse model of human MS.

All experience of significant pain (for instance during wounding) will be under anaesthesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Mild 80%, includes animals used for:

Breeding colony for GA strain

Short term immunisations or mild infections

tumour experiments

chronic tissue remodelling

Moderate 20%, includes animals used for:

infections intended to introduce wounding or mice with induced auto-immunity. These mice may experience weight loss till about 20%

in EAE (the mouse model of human MS) experiments animals may experience transient paralysis

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Pathological processes, that often underly diseases, progress over a very long period of time. During this time, a wide range of different cell types need to communicate with each other. The immune system is key player in these pathological processes. Nevertheless, the immune system itself is yet again a highly complicated network of different cell types that at different time points secrete distinct patterns of factors that all influence each other.



In particular, key players of the immune system, so called T-cells, are activated at locations that are physically separated from the site of inflammation or infection. Pathogen derived products are actively transported to these sites, where T-cells are activated, and the these activated T-cells return to the site of inflammation / infection, where they perform their protective function. These functions can then lead to pathogen clearance but may also contribute to wound healing and the maintenance of tissue well-being. Thus, while in the last decades we have made tremendous progress in understanding the underlying processes of this system, we are far away from being able to replicate these processes *in vitro* or in computer simulations. Thus, only animal experiments will allow us to thoroughly address these issues.

What was your strategy for searching for non-animal alternatives?

A substantial part of our research will be performed using *in vitro* model systems. For instance, we perform T-cell cell differentiation in a dish to understand the underlying mechanism that contribute to their differentiation. Also, single cell sequencing of T-cells is performed, which allows us to determine the affinity of T-cell receptors *in vitro*. Furthermore, mutant cell lines are established that simulate mutations induced in mice. These cell lines can then be tested for change in established cell biological mechanisms.

We use these model systems like these to establish and test specific hypotheses, that can then be tested in animal experiments for their validity.

Why were they not suitable?

These *in vitro* model systems are very valuable to define and test some hypotheses, in specific on a single cell level. Nevertheless, pathological processes are highly complicated and the interplay of several different cell types determines the disease outcome. Thus, in order to determine the physiological relevance of a novel immune modulatory process appropriate mouse experiments will have to be performed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Only a fraction of these mice will undergo an experimental procedure. Most animal will be used for breeding. Experience from the last 5 years suggest that this is about the number of mice we need to keep our mouse colonies going.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We normally plan exactly, when experiments can be performed. In this way, we very efficiently can use every single mouse born. Given a mouse cannot be used for *in vivo* experiments, then these mice are normally used for *in vitro* experiments.



In addition, for many tumour experiments, we moved to in vivo detection of tumours, using luciferase. This allows us to track tumour growth and responses to our experimental manipulations in one and the same mouse. So far, we always had to cull test groups at different time points of an experiment. In this way, we can save several test groups. Furthermore, use to more precise measurements, this method may also allow us to reduce each experimental group as well.

What other measures apart from good experimental design will you use to minimise numbers?

We very carefully manage your breeding colonies in response to experimental plans to reduce wastage. Excess bred mice might be killed and cells and organs be used for in vitro experiment or may be used to carry out pilot experiments to optimise dosing/new protocols to maximise data output.

Any new developments in the field we will adapt to be able to diminish animal usage.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will follow and characterise immune responses induced in several different ways. All proposed procedures follow world-wide standardised methods, which have been refined extensively over the years.

In this way, the standardised read-out systems can reliably reveal and explain underlying mechanisms of a process, while causing minimal distress to the animals.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The immune system has developed in vertebrates, thus lower animals would not suit for our studies. Furthermore, the immune system in mammals is substantially more advanced than the one in, for instance, fish. Thus, the lowest mammal species is required for our studies, with the mouse being an excellent and well studied model system for which a wide range of standardised read-out systems have been established.

With regard to sedation, immune responses are usually studied for between 5-15 days and pathology is normally studied in models of chronic exposure. No animal can be under sedation for such a long period of time.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Over the last 20 years, we consistently have been refining our work.



For example, the majority of animals will be euthanised before showing clinical disease symptoms; less virulent pathogen strains feature more frequently in our experiments; and we have improved the ways that we administer drugs and blood sample infected animals. Furthermore, we avoid single-housing mice so they can benefit from huddling and social interactions.

We are also continually refining our endpoints as we gain more experience with different experimental set ups. Furthermore, animals at critical time points of specific sets of experiments are monitored at least daily and often around-the-clock during symptomatic periods, so that measures can be taken to facilitate their recovery. If animals show signs of sickness they are given supportive nursing measures (e.g. mash, transgel).

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The REDACTED regularly issues guidelines for how to improve experimental procedures.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

REDACTED communicate advances and development opportunities regularly.

Furthermore, we are in close contact with researchers worldwide, who keep us informed on developments in this field.

In addition, we feel it is imperative to communicate closely with animal care technicians to inform the monitoring of animals and how to make interventions more effective. We will further invest into this line of communication.

Explain the choice of species and the related life stages

Mice are an exceptionally good model system to understand the functioning of the immune system and the contribution of the immune system to pathology and disease. Major breakthroughs in cancer immunotherapy, which currently heal thousands of people suffering from cancers, have first been achieved in mouse experiments. This has been achieved thanks to the uniquely characterised biology (e.g. immunity and physiology) of lab mice. Murine immune responses are well defined and the technology is highly developed that enables sophisticated manipulations of the immune system and of individual cell types that contribute to its functioning.





182. Understanding phenotypes resulting from imbalances in protein O-GlcNAcylation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

No answer provided

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 is the aim of this project?

We will study the molecular mechanisms underpinning the intellectual disabilities seen in people carrying mutations in genes that control the modification of certain proteins by the addition of carbohydrate components.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Intellectual disability affects ~1% of the world population and its causes are only beginning to be understood. This work will results in an increased understanding of a sub-type of this disease paving the way for future development of therapeutics.

What outputs do you think you will see at the end of this project?

The primary benefit from this project will be novel information of this particular class of protein- modifying enzymes and their substrates within biochemical pathways. Our insights in this area to date have had significant translational impact, through the development of a



number of in vitro assays/inhibitors which are now in use by a number of drug companies in their pre-clinical programmes targeting these enzymes in neurodegenerative diseases.

In addition to the direct scientific benefits, we perform patient and public outreach. For example, we have presented our result at a forum attended by patients, to explain the science that we perform and how this adds to the knowledge of their disease. The close scientific links between our group and clinicians in Scotland and Europe will facilitate patients coming to REDACTED from nationwide to better understand their condition.

Although the generation of REDACTED is time-consuming and expensive, the clear-cut highly reproducible answers which stand the test of time that this approach delivers makes the effort worthwhile. Furthermore, mouse studies on these new potential targets will lead to new interactions and collaborations with other researchers, clinicians and pharmaceutical companies who will use our models in their own analysis to drive the research field forward. We will share our animals, reducing the need for others to generate lines and therefore reducing overall animal use.

The key aim of our mouse work is to use knock-in or knock-out mice in which the modification of proteins by carbohydrate components is disrupted and use these in conjunction with other research to provide a robust framework of knowledge of how the signalling pathways are regulated and organised. We also wish to understand how these pathways intersect with others that appear to be involved in Parkinson's and Alzheimer's diseases.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The results from this work would indicate that patients bearing these mutations/suffer from these neurodegenerative diseases might benefit from a future drug that targeted OGA and this information would be useful for pharmaceutical companies developing OGA inhibitors.

How will you maximise the outputs of your work?

Primarily the new knowledge generated will be published in open access journals. REDACTED generated will be made available to others in the field to maximise output from them. Even where the original hypotheses are rejected we will publish these data in a peer reviewed scientific journal.

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.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.



Mice carrying patient mutations will be generated using genetic approaches. Tissues derived from these mice will be used for biochemical analyses. Mice will undergo behavioural assays aimed at testing memory/habituation functions in a non-stressful setting. Some animals may receive substances that are intended to affect the biochemical pathways under study.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We will use established mouse models of neurodegenerative disease and generate new models that carry the same mutations as patients suffering from specific inherited intellectual disabilities. We will study the molecular changes that occur in cells and tissues as a result and will correlate these findings with behavioural and cognitive testing in the mice. We may administer agents that are already known to affect how the enzymes of interest work in isolated cells. As neurodegenerative disease in humans is usually associated with ageing, we expect to have to keep some animals at least for one to two years, in order to be sure that we would significant significant changes in 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 species)?

We do not expect any animal to become seriously unwell.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

While we can introduce genetic changes of interest into previously “normal” cells, we cannot recapitulate the complexity of the human (or mouse) nervous system that might translate a change at the DNA level into a behavioural difference, without using an intact animal.

What was your strategy for searching for non-animal alternatives?

We already use cell lines and the fruit fly as models where possible.

Why were they not suitable?

There are no suitable “Alzheimer’s” or “Parkinson’s” cell lines in which to study whether the enzymes of interest are affected in these human diseases.



Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Breeding and maintaining the required number of lines, with the final numbers being dictated by the genotypes obtained.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Hypothesis-testing experiments will be designed to have sufficient statistical power to be robust, without using too many animals.

What other measures apart from good experimental design will you use to minimise numbers?

Breeding and maintenance will be carefully managed so as to prevent waste.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use genetically modified mice.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We have chosen to use mice as they are easily genetically standardised and modified. Their nervous systems are more similar to our own than, for example, the fruit fly system is.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We expect the great majority of animals to experience very near-normal welfare but will implement scoring systems to ensure that any adverse effects of neurological changes are recognised and kept to the minimum consistent with the scientific objectives.



What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow the LASA guidelines on administration of substances.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will regularly consult the NC3Rs website and participate in local animal use discussion platforms.

Explain the choice of species and the related life stages

Mice are genetically tractable, have a short generation time and a gross central nervous system similar to our own.





183. Understanding platelet function and regulation in a Zebrafish model

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants.

Key words

Zebra fish, Platelet, thrombosis, haemostasis, development

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

Platelets are small cells in the blood that play an important role in the prevention of bleeding. However, platelets can also stick together to form a clot, called a thrombus, which can result in a blocked blood vessel. This can reduce blood flow to the heart, leading to a heart attack, or to the brain, leading to a stroke. Platelets have also been shown to play an important role in the interaction with other types of cells. Currently treatments which influence the behaviour of platelets are not effective for all patients and some patients experience life threatening bleeding. This programme of work aims to understand how platelets work to identify new and safer targets for patients. The programme will also enable judgments to be made of potential side effects of drugs with influence the interaction of platelets with other 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.

What are the potential benefits that will derive from this project?

This project will advance our knowledge of platelet function and how this can be manipulated to develop new and safer ways to treat patients at risk of heart attack or stroke. By understanding platelet function, a better understanding of potential side effects



from drugs which influence platelet function and the interaction of platelets with other cells can be achieved.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

We expect to use 12500 adult Zebrafish over 5 years. Where possible the larval form, before the onset of independent feeding, will be used

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Most of these animals will be used for breeding only (mild severity) and it is expected that there will be little or no adverse effects from breeding. Some animals will experience minor procedures such as small blood samples. Most experiments will be conducted under anaesthetic from which the animals will never wake up from. Some animals may be exposed to substances in the water or occasionally by other routes, before being anaesthetised for the procedures. The animals will then be humanely killed. We do not expect the animals to experience more than minor distress from these procedures. Any animals which show more than minor discomfort will be humanely killed.

Some of these animals will be kept until they reach the end of their healthy lifespan (usually between 2 and 3 years). These animals may be exposed to substances in the water and be imaged non-invasively under anaesthetic at intervals. Once these animals display signs of distress they will be humanely killed.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

How platelets respond to injury and contribute to developmental processes cannot be understood without the use of animals. It is not currently possible to recreate the complexity of these processes with alternative approaches which do not need to use animals. The majority of experiments we will do in this project will be in Zebrafish larvae. The majority of protected animals will be used to breed fish with genetic modifications

Reduction

Explain how you will assure the use of minimum numbers of animals.

We will use the literature and discussions with others with relevant expertise to ensure we are using the minimum number of animals. Animals will be used for breeding only over



their healthy lifespan. The literature and data from pilot studies will be used to predict the group sizes needed to generate statistically significant data.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Zebrafish are used to identify and understand genes which affect human development and diseases as many genes and cell biological processes are found in both humans and Zebrafish. Zebrafish have many of the genes important for platelet function and genes which mediate the interaction of platelets with other cell types. Where possible substances which influence platelet behaviour will be given in tank water removing the need for physical administration. Anaesthesia will be used to minimise any discomfort experienced by the fish and fish will be continuously monitored while under a procedure.





184. Understanding the behaviour and welfare of ruminants

Project duration

5 years 0 months

Project purpose

- 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

No answer provided

Animal types	Life stages
Sheep	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 is the aim of this project?

Farmed ruminants can experience poor welfare from many sources: painful management procedures, early mother-offspring separation, artificial rearing, inadequate environments, among others. The aim of this project is to develop a better understanding of the impact of these interventions on behaviour and welfare, and to develop management practices to improve or mitigate welfare challenges.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Ensuring the welfare of all farmed livestock species is an important part of ethical and sustainable food production of animal origin. Historically, whereas there has been a lot of attention given to the welfare issues affecting very confined animals, especially pigs and poultry, rather less attention has been directed towards ruminant species, particularly as they may often be relatively less confined. However, there are a number of management practices that can have a very significant impact on ruminant welfare, where information on the impacts and best practices are still being determined. Currently the separation of



very young animals from their mothers, particularly in dairy animals (sheep, cattle and goats), are significant welfare challenges in ruminant production. The work outlined here will address these issues, and seek to develop novel or alternative practices that can improve ruminant welfare, by providing objective scientific evidence of the short and longer term impact of these management practices. These data will lend scientific rigour to discussions and debates around what can often be emotionally charged discussions on animal welfare.

What outputs do you think you will see at the end of this project?

We would publish all new information in peer reviewed journals, and make the data available to other researchers on request, and present our work at conferences. We would anticipate a minimum of 10 published papers as a result of this work. As the aim of these studies is to improve on farm management we will also present the work to stakeholders (farming community, farmer organisations, etc) and work with agricultural consultants to disseminate the work. We will make use of existing networks and platforms to share the information to farmers, veterinarians and advisers across Europe.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

The beneficiaries of this work will be:

Animal welfare and other scientists - who will gain further knowledge on the impact of the mother on the development of young animals. This will inform other research in other species (e.g. dairy cattle), and contribute to the body of work on early life impacts on development. The work will also develop standardised tests, which will have wider benefit for application in assessments of animal responses in other experimental settings. These benefits will be achieved throughout the lifetime of the project.

Farming community - who will gain information on optimal husbandry strategies for managing young animals, particularly for those using artificial rearing. These benefits are likely to be realised towards the end of the project when the complete data are available.

Farmed ruminants – the aim of this work is to improve the welfare and management of young farmed livestock, resulting in improved quality of life for ruminants in the future. The knowledge and understanding of the issues may also be relevant to other species, where early weaning and raising without the mother may also occur, such as rearing of surplus piglets.

Policy makers - the information developed will be of use to policy makers in the formation of animal welfare and food policy to develop ethical practices for the treatment of farmed animals. We will work with government and other agencies throughout the project to keep them apprised with the work, and benefits could be achieved throughout the project.

Retailers - ethical food production is increasingly on the agenda, and consumers have become more concerned about the way animals are treated on farm. The work will be of benefit to retailers seeking to respond to this concern by providing informed evidence on which to base decisions.

How will you maximise the outputs of your work?



We will continue our collaboration with scientists in REDACTED, who are working on brain imaging techniques which informs the behavioural responses we would expect to see, and develop new collaborations where appropriate. New knowledge will be disseminated through academic channels and news-worthy outcomes will be disseminated via press releases and articles to allow uptake by the general public. Animal behaviour, including of farmed animals, is often of interest to the public and we will aim to make the work widely available. We will use as many of our existing channels as possible to reach policy makers, farming organisations and retailers to ensure that they have access to good quality evidence on the impact of husbandry practices on animal responses. We will also share the behavioural testing paradigms used widely with others in the scientific community, including information on those aspects of the work that have not been successful.

Species and numbers of animals expected to be used

- Sheep: 240
- Goats: 24
- Cattle: 24

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

Typically animals in the project will be separated from their mothers between 24 and 48 hours of age, mimicking what occurs in the dairy industry for different ruminant species. The impact of this early life event on subsequent emotional and neurological development will be assessed through the use of behavioural tests that ask how well the animal can learn different tasks, or how they respond to pleasant and unpleasant events. These tests are designed to be relevant to the species and would be similar to, or not exceed in unpleasantness, the normal practices of rearing these species on farm. To help us understand the full impact on the animal we will combine behavioural outcome measures with sampling of blood, saliva or faeces to build a complete picture of how early life events influence animal development.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

We anticipate that rearing without the presence of the mother will alter neurological development in young ruminants, and will be associated with distress on initial separation. This may not have long lasting impacts on the animal when maintained in benign and stable conditions, but could increase distress, anxiety and fear when animals are exposed to frightening or challenging stimuli, and may also limit the ability of the animal to experience pleasant responses. In addition, this may impair the learning ability of lambs, calves or goat kids, which could lead to feelings of frustration or anxiety. Rearing without the mother may also reduce immune function, which increases susceptibility to disease, and cause greater responsiveness to pain and 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 species)?

We anticipate that the severity of the procedures to be used will be mild, and for nearly all the animals used in the project. Our aim is to mimic commercial on farm practice throughout, and as far as possible we will use behavioural tests that rely on animals learning through rewards and pleasant experiences rather than negative events.

What will happen to the animals at the end of the study?

- Kept alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal welfare is about the experience of an animal: its feelings and how it copes with the environment. We use animal behaviour as a key indicator of how the animal is experiencing its environment. It is not possible to replicate this in a non-animal model.

What was your strategy for searching for non-animal alternatives?

Computer-based modelling studies may allow us to make some predictions about how animals might cope with environmental changes.

Why were they not suitable?

These methods are useful to help refine and improve design, but to be able to access animal welfare measures there is no suitable substitute for the use of 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?

Numbers were estimated based on our previous experiments to derive data for power calculations, or from the literature.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?



We have used advice from our local statistical consultants in designing the studies to minimise the numbers of animals required, and drawn on the expertise of our collaborators and our own experience of running similar experiments to optimise the numbers of animals required for the project.

What other measures apart from good experimental design will you use to minimise numbers?

Where the variance of some behavioural outcome measures is less well known, or the impact of early life experience on these measures is less well studied, we will monitor animal numbers and effect strengths to

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We will use the newborn offspring of sheep, cows, or goats, from birth through to weaning and until they reach adulthood for some. These animals, and their mothers, are most commonly used on farm in normal commercial production, and we need to know how they respond to various challenges in order to ensure that we can improve their management to reduce suffering and improve on farm welfare.

The main methods we will use will be behavioural observation. This may be by observing and quantifying spontaneous behaviour (such as maternal care) or by imposing various behavioural tests on an animal - such as responses to novel objects, ability to solve spatial or other puzzles, discriminatory abilities, startle or judgement bias tasks. These may require minimal unpleasant experiences of the animals (e.g. exposure to novelty can provoke fear in some animals) but as far as possible these will be paired with neutral or pleasant experiences.

For some parts of the study we will replicate procedures typically conducted on commercial farms (such as early mother-offspring separation as used by the dairy industries). These may be associated with fear, or distress, but to ensure that we can understand the impact of these procedures on behaviour or other biological outcomes, and in order to be able to develop improved methods or advice, this is unavoidable. As far as possible we will ensure that we make use of appropriate protocols to minimise suffering, and will apply rigorous endpoints and interventions in our management.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

The work will assess the behavioural and cognitive impact of on farm procedures - this cannot be achieved by using other species or life stages, as farm management is specific to species, and the impact of these procedures may be less important or less long-lasting in other animals. Maternal care differs between species, and the impact is also dependent



on the stage of development of the offspring at birth (for example some animals may be very immature at birth, whereas others are considered to be precocious). Although studying maternal care in other species can be useful for developing hypotheses, for the work to have application in improving animal welfare it must be conducted on the relevant farm species.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

We will monitor the impact of early mother-young separation on our outcome measures, and adjust these if the severity is greater than anticipated. Should we find that the impact on neonatal development leads to greater fear and distress in the testing process than we expect we will modify test design to reduce the fear-related components. Animals will be trained and habituated to testing and sample collection to reduce the impact of fear of novelty or restraint. We will use pain relief and rigorous pain management where ever this is feasible, and will aim to shorten the period of pain assessment to the minimum required to achieve experimental objectives.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will use NC3Rs guidance on positive reinforcement training, handling and restraint, anaesthesia and analgesia where relevant. We will also adhere to published guidance on best practice in behavioural testing from the Laboratory Animal Science Association (LASA). Animal housing and husbandry will at least meet, and may exceed, that required under the ASPA.

When publishing our work we will use the ARRIVE guidelines to ensure that we make all the relevant information known to others to help refine further studies.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will continually review our procedures throughout the time course of the study, and seek less harmful alternatives whenever these are available at birth, or maternal strategies, make extrapolation between species unreliable. Therefore, we need to study these species to be able to understand the full impact of early mother-young separation. In order to develop improved methods to manage young ruminants, and to mitigate any potential adverse consequences, we need a better understanding of the impact on neonatal animals, and through to adulthood, of early life events.





185. Understanding the mechanisms underlying chronic obstructive pulmonary disease (COPD)

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 is the aim of this project?

The aim of our work is to better understand the processes driving chronic obstructive pulmonary disease (COPD). Despite much research to date, we still do not understand what triggers and drives disease forward well enough to develop better therapies to stop or reverse this – current therapies merely help manage the disease, and this represents a huge unmet clinical need.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

COPD has recently emerged as the third leading cause of premature death globally (World Health Organisation data). COPD causes an enormous health and socioeconomic burden, and this burden is growing due to the lack of research into better treatments, when compared to e.g. cardiovascular disease, where mortality is falling because intensive research has led to better treatments for patients.



The processes that drive COPD are poorly understood, but chronic inflammation is clearly very important. This Project focuses on the role of a cellular protein, REDACTED, which we know plays a role in other lung diseases, and is a key driver of a range of diseases that feature inflammation. This suggests REDACTED could be an attractive drug target in COPD, and this work will test whether blocking REDACTED activity could be a valid therapeutic strategy to develop drugs for more effective COPD treatment.

What outputs do you think you will see at the end of this project?

This work is expected to provide new insights into how the lungs can be regulated. It will provide us with an understanding of the pathways that contribute to the perpetuation of disease and repair processes, which are associated with chronic obstructive lung disease. The development of novel therapeutic strategies requires an advanced understanding of the roles and mechanisms of action of cells and proteins that are altered in disease.

This work will help provide that, and the new information we discover will be disseminated as widely as possible to other researchers via scientific publications (in journals and at scientific conferences), so they can build on this knowledge.

We will also widely engage with the public, both directly (e.g. school visits) and through social media, to help educate more people about the importance of maintaining healthy lungs. The important long-term benefit of this work is that these studies will help identify and validate new approaches that can be used to treat or better manage COPD, and alleviate the burden of disease and disability.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short to mid-term term (within Project timescale), the Project will benefit REDACTED researchers including partner organisations, with a focus on understanding the disease process in COPD. In the medium term, the new information and insights about COPD will be beneficial to researchers internationally (academic and industrial) who have an interest in lung disease and inflammation in diseases, because we will publish our findings in open-access journals so that others can follow our work or use our data to inform their own research.

We anticipate that in the long term, this Project will benefit COPD patients, since there is currently significant interest and progress in developing REDACTED-targeted compounds as future drugs to tackle disease, and our research could highlight that COPD may be one such important therapeutic avenue.

Our public engagement work, which arises directly from our scientific research, will also benefit COPD patients in REDACTED in spreading greater awareness about their condition and state-of-the-art research that is underway to help them. These activities will also benefit schoolchildren in this region, as greater awareness of the importance of lung health and prevention of tobacco product abuse will contribute to future health outcomes.

How will you maximise the outputs of your work?

REDACTED

Species and numbers of animals expected to be used



Mice: 5,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

This project is very simple and does not involve procedures required to replicate or 'model' a human disease in an animal. Instead, we will answer our scientific questions by using animal cells and tissues after their death, via sacrifice of the animal by a humane method. However, since we need to compare the results of experiments using both normal (wild type) and genetically altered (for example lacking REDACTED) animals, we do need to breed and maintain these animals in the laboratory, and to test them to see if they are normal or REDACTED-deficient (by painlessly removing a piece of ear tissue).

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

The procedures are all related to the breeding and maintenance of the animals, and classified as mild in severity. Once culled humanely, appropriate tissue/organs/body fluids will be harvested for the purpose of laboratory experimentation.

Therefore, possible adverse impacts relate only to the housing of animals, testing whether they are normal or genetically altered, and maintaining them in the laboratory for periods up to 15 months.

We do not expect adverse effects on the animals as part of this project, but we will monitor the welfare of animals on a daily basis, and ensure that any unexpected discomfort or suffering is ended as quickly as possible, by humane killing if necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

The procedures are all related to the breeding and maintenance of the animals, and all expected outcomes are classified as mild in severity. All of the animals are expected to be classified as Mild.

What will happen to the animals at the end of the study?

- Killed

Replacement



State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Genetically altered animals allow us to investigate definitively the effect of a molecule we think may be a good drug target – by testing the way cells and tissues of the body function in the absence of that protein.

The reason animals are required for these experiments is due to the multidimensional nature of lungs and therefore lung disease. It is not just certain types of cells (for example airway lining or muscle) that contribute to disease, but how they interact with each other in the complex internal environment of the lung. It is often not possible to duplicate these interactions in anything other than that of intact mammalian tissue, and our experiments will involve using pieces of lung tissue that incorporate these complex interactions.

What was your strategy for searching for non-animal alternatives?

Human cells and cell lines will be used wherever possible, to complement and validate our experiments that use mouse tissues. However, human lung tissue is not available in amounts needed to answer our scientific questions regarding complex interactions that occur between different types of cell within the lung, therefore we need to use mouse tissues as a model for the human organ.

In addition, human tissue that is genetically altered to lack certain molecules we need to study is not available, therefore using genetically altered mice, which are readily available, is the best way for us to selectively study the effect of molecules such as REDACTED.

Why were they not suitable?

As explained above, the reason animals have to be used in these experiments is due to the multidimensional nature of lung disease. It is not just particular cells that contribute to disease, but interactions between many different cell types in the particular arrangement and structure we see in the lung. It is not possible to duplicate these interactions in anything other than that of an intact mammalian system. Also, we cannot study the impact of lack of important molecules such as REDACTED in human tissue, since this requires genetic alteration, not feasible in humans but already performed in mice.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

To ensure the minimum numbers of animals are used we will only use sufficient animals necessary for valid statistical analysis (so that our results are clear and reliable). We have estimated the numbers of animals required based on size of effect that we have seen in pilot studies, and using well-accepted statistical prediction models based on these



observations. A dedicated BREATH statistician is involved in aiding design and analysis of experiments.

Experiments will be performed on cells, tissues and organs collected from humanely killed animals, rather than using mouse models of disease that involve multiple licensed procedures on living animals. Not only does this mean that we can address our scientific goals in a project that does not cause significant harm to animal welfare, but also means we can minimise animal use by often using multiple cells or tissues from each animal in different experiments.

Efficient breeding strategies (generating only genetically altered, and not normal animals) will be explored as a further approach, to reduce animal numbers used. However, we must first test that this does not compromise normal physiology (e.g. inflammation responses) of the cells and tissues, and therefore experimental outcomes. Thus, we have not incorporated number reduction assumptions that are dependent on such approaches into the estimate presented here.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We have already designed this Project based on not only a significant body of scientific literature, but further on the basis of convincing pilot studies in our laboratory, using surplus animal tissue accessed according to 3Rs principles.

We have used data from pilot studies to ensure we can measure clear results in our experiments, and performed statistical calculations in order to reduce our estimates of animals needed.

We will continue to liaise with a dedicated statistician, formally linked to the Project, throughout to ensure that experiments are designed efficiently.

We will also use NC3Rs Experimental Design Assistant tool where appropriate, and refer to appropriate recommended experimental design texts.

Finally, we will use the information obtained from our animal experiments to inform our experiments using human cells, and vice versa. This will ensure that we only perform the most scientifically useful experiments, and reduce the overall number of experiments that require animal tissues.

What other measures apart from good experimental design will you use to minimise numbers?

Our estimate of animal numbers required is based on a breeding strategy that generates normal animals (wild type) as well as those genetically altered by full or partial loss of a gene function. Although this provides the different types of animals we require to make scientifically meaningful comparisons, it is possible that we may not need as many 'normal' comparators as the genetically altered animals (because 'normal' tissues may serve as controls for several experiments). In this case, we will explore whether it is possible to reduce the number of animals required for breeding by mainly pairing genetically altered animals. This alternative approach could potentially reduce animal numbers used, provided this does not compromise normal behaviour of the animal cells and tissues, and therefore experimental outcomes.



We will inform other workers within the Institution (and external collaborators) when culling animals, so that surplus tissues can be made available to other studies (external to this Project) according to 3Rs principles. An example of this would be knee joints, which could be used to provide 'control' standard tissues for ongoing work within our Institute focused on joint disease.

Importantly, we will perform ongoing weekly review of study data generated, to ensure experimental objectives are reached and thus stopped at the earliest possible checkpoint.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

The current project will use tissues from normal 'wild type' and genetically altered mice, a model widely used in the scientific community. Our laboratory experiments will use cells and tissues taken from animals following humane sacrifice. Prior to this, we will breed and maintain the mice for a period of up to 15 months in our licensed facility, and will take small biopsies (usually by painlessly removing some ear tissue) to identify the mice as normal or genetically altered. We therefore expect that there will be no significant animal suffering associated with this project, and we will monitor animals in our care carefully and daily to minimise any unexpected harms. Any unexpected suffering that may occur will be minimised by pain relief where appropriate, and comfort will be maximised by measures such as increased bedding, ensuring warmth and providing easy access to food and water.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

We must use mammals for this work, since the physiology of other animals would be too far removed from that of humans to generate any data that would be instructive to the study of human disease.

Mice are the small mammal model of choice due to the availability of genetically altered animals with particular characteristics that enable researchers study the importance of particular molecules and genes, to test whether these molecules and genes would make good targets for new drugs to treat disease. In this case our major focus will be on testing whether a molecule called REDACTED is likely to be a viable drug target for COPD.

Since COPD is a disease that develops in humans over a long period and is associated with maturity/advancing age, it is important that adult, fully developed animals are used for this study. We will maintain mice in the laboratory up to a period of 15 months, approximating to human middle age.



Only procedures associated with breeding will be used in this project, minimising potential for significant animal suffering. Tissues and cells will be harvested and utilised in experiments after humane sacrifice by an approved method.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

Significant welfare costs to animals are not expected in this project, since only procedures associated with breeding and maintenance of normal and genetically altered mice are included. Health of animals will be carefully monitored by experienced staff on a daily basis, and we do not expect significant adverse characteristics of the genetically altered animals, with which we already have considerable experience.

For genetic testing, techniques with least impact on animal welfare will be utilised will be used to minimise possible infection,

Mice kept to middle age (up to 15 months), could show signs of ageing, such as skin sores (which will be resolved with pain relief creams), or excessive growth of teeth (which will be clipped if necessary).

Other refinement measures will include environmental enrichment, increased bedding, maintaining warmth and easy access to food and water.

If welfare costs are greater than expected as normal for a mouse of this age, then such animals will be humanely culled by staff trained and assessed as competent.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

The NC3Rs website hosts a wide range of resources, including online videos, dedicated to refinement through such means as optimised experimental design, and housing/husbandry.

<https://www.nc3rs.org.uk/3Rs-resources>.

This site will be accessed regularly to search for updated resources. The applicant is registered with NC3Rs in order to receive regular newsletters and email updates, and the Named Information Officer also provides updates to users of the animal facility and to AWERB.

NC3Rs strategy for improving welfare of research animals is published:

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/lab.an.1217.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

The applicant is registered with the National Centre for the 3Rs (NC3Rs) to receive email updates on new advances, and will also receive information through the Named Information Officer, part of the Animal Welfare and Ethical Review Board (AWERB) of the REDACTED.



Effective implementation of new advances will be discussed at regular (monthly) meetings where researchers involved in use of animals assemble, and at AWERB where appropriate. Project staff will be trained in new advances so that these can be integrated into the normal work pattern of the project.

Explain the choice of species and the related life stages

The project will use mice, from common laboratory bred strains.

The reason that we are using mice is because, as mammals, their physiology (how the body works) is broadly similar to that of humans, so that we might expect lessons learned by using these animals are likely to be relevant to humans and human disease.

Another important reason to use mice in this work is because laboratory mice that are genetically altered to lack a certain molecule, are available to study. Such mice are extremely valuable to scientific investigation, as they allow us to study the impact of the absence of a specific molecule, normally present in cells, has on how the cell, tissue, organ or body system works. This is very important in predicting the outcome of blocking the molecule in question using a drug. In this case we will be mostly working with mice that lack a molecule we have found to be important in inflammatory diseases such as arthritis, called REDACTED.

We will use mice at adult stages, from maturity to advanced middle age. We will use these stages since these are the periods during a human lifespan that people are likely to develop, then show signs and symptoms of COPD.

We have not experienced or are aware of any adverse effects to the animals of lacking REDACTED in our project, but will monitor them daily in order to ensure any possible unexpected suffering is minimised. The project will use mice, from common laboratory bred strains.

The reason that we are using mice is because, as mammals, their physiology (how the body works) is broadly similar to that of humans, so that we might expect lessons learned by using these animals are likely to be relevant to humans and human disease.

Another important reason to use mice in this work is because laboratory mice that are genetically altered to lack a certain molecule, are available to study. Such mice are extremely valuable to scientific investigation, as they allow us to study the impact of the absence of a specific molecule, normally present in cells, has on how the cell, tissue, organ or body system works. This is very important in predicting the outcome of blocking the molecule in question using a drug. In this case we will be mostly working with mice that lack a molecule we have found to be important in inflammatory diseases such as arthritis, called REDACTED.

We will use mice at adult stages, from maturity to advanced middle age. We will use these stages since these are the periods during a human lifespan that people are likely to develop, then show signs and symptoms of COPD.

We have not experienced or are aware of any adverse effects to the animals of lacking REDACTED in our project, but will monitor them daily in order to ensure any possible unexpected suffering is minimised.





186. Understanding the progression of hypertensive heart 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

No answer provided

Animal types	Life stages
Rats	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What is the aim of this project?

To investigate the role of inflammation and elevated sympathetic nerve activity in the progression of hypertensive heart 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?

Heart disease is a major cause of ill health and the leading cause of death worldwide. Hypertension (consistently and abnormally elevated blood pressure) is a major risk factor of developing heart failure. Despite heart failure being a debilitating and fatal condition, the mechanisms involved in the progression of hypertension to heart failure are not well understood. This study aims to investigate the role of inflammation and sympathetic nerve overactivity in the progression of hypertension into heart failure. Understanding the causal



mechanisms of the disease will allow identification of new therapeutic targets that will prevent disease progression. Furthermore, this study should help identify early markers of disease progression enabling early intervention. To achieve these goals, the study will combine research in humans and animal models of the disease.

What outputs do you think you will see at the end of this project?

We expect that this study will provide new information on the factors contributing to the progression of heart disease in response to high blood pressure. We expect the study will provide markers for the early detection of patients who are likely to progress into heart failure and require intervention (i.e. new diagnostic approaches). Finally, we expect that we will identify potential new treatment targets that could be used to prevent or slow disease progression. Depending on study outcomes, we expect to provide pre-clinical proof-of-concept data that will allow the repositioning of licensed drugs. Our findings will be published in open access PubMed-indexed peer-reviewed outputs.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

Immediate benefits to scientific knowledge. The work carried out under this license will advance fundamental scientific knowledge of the progression of hypertensive heart disease. Our findings will be made available to other scientists and clinicians through publication in peer-reviewed journals and presentations at scientific conferences. We also aim to publish (using self-archiving tools) full datasets of the parameters measured in the disease models, for re-use by other scientists. In doing so, the study will provide a rational pathway for the discovery of new therapeutic targets. It will also provide further clinical validation of animal models of high blood pressure. The study will also provide educational value by training a PhD student in pre-clinical and clinical approaches to medical research. We also aim to disseminate our findings to patients and their families via outreach activities.

Future potential benefits to patients. Long term, this project could be the first to deliver a safe and efficacious pharmacological treatment to prevent the progression of hypertension into milder variants of heart failure. This would benefit patients through improved health, quality-of-life and lengthened life- expectancy and reduce the socioeconomic burden caused by this disease. The likelihood of success is high, because during a previous programme of work, using the same animal model, we identified a new therapeutic target for treatment of drug-resistant hypertension that was subsequently validated in humans.

How will you maximise the outputs of your work?

As mentioned above, our findings will be published in open access PubMed-indexed peer-reviewed journals and will be presented at scientific conferences including clinical conferences. We also aim to publish (using self-archiving tools) full datasets of the parameters measured in the disease models, for re-use by other scientists. Lastly, we will contact the REDACTED media team to advertise published findings to a broader audience.

Species and numbers of animals expected to be used

- Rats: 160



Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

For approximately one third of the animals, the experimental studies will only involve the induction of a general anaesthesia. They will then be killed to enable the harvesting of tissue for analysis, never recovering from anaesthesia. Two thirds of animals may undergo one surgery under general anaesthesia and appropriate analgesia. Surgery will be performed using a strict aseptic technique and the animals will be given pain killers until they fully recover. Some animals may experience mild transient discomfort from drug administration and/or transient restraint for non-invasive measurement of blood pressure and blood sampling on more than one occasion. On separate occasions, some animals may undergo temporary sedation/anaesthesia to allow non-invasive measurements of cardiac function. These experiments are expected to last 12 weeks or less.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Surgery under general anaesthesia and appropriate analgesia, is associated with post-surgical pain, and transient suffering or impairment of general condition. Most animals are expected to make an unremarkable full recovery from surgery within 3 days. Experiments are not expected to interfere with the animals' natural behaviours, some may cause only minor transient discomfort or stress, but are not expected to cause lasting suffering or harm. All animals will be killed humanely at the end of the study to enable tissues to be harvested for further analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per species)?

Approximately, one third of animals are expected to undergo mild severity, the remaining are expected to undergo moderate severity.

What will happen to the animals at the end of the study?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?



Rodents are the lowest animal order in which control of blood pressure is like humans.

What was your strategy for searching for non-animal alternatives?

A search of ATLA (<http://www.atla.org.uk/>), ALTBIB (<https://toxnet.nlm.nih.gov/altbib.html>) has returned no alternatives for achieving our experimental goals. We have considered humans, non-protected species, *in vitro* models (including organoids) and computer models.

Why were they not suitable?

Control of blood pressure takes place in the brain, one of the most complex organs. High blood pressure is believed to result from a combination of numerous factors. Thus, *in vitro* methods cannot reflect this complexity. The causes of the condition addressed in this project are not fully known and cannot be simulated in a computer model. Rodents are the lowest animal order in which control of blood pressure is like humans. Even though we will use humans in this research, the current technology available for human research is not refined enough to reveal the complexity of interactions we aim to study. Additionally, human studies can only establish correlations not causality; some of the tissue samples and interventional procedures required to confirm causality cannot be obtained/performed in humans. For these reasons, there is no replacement for use of animals in this project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We carried out power calculations based on our data and published data.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

Experimental design and analysis were planned and discussed to prevent unnecessary replications. Clinicians, clinical physiologists, patient advocates and Pharma partners were consulted to ensure that data can support future clinical trials. Advice was also sought from mathematicians and statisticians.

What other measures apart from good experimental design will you use to minimise numbers?

We will measure as many outputs as possible from the same animals, particularly *ex vivo*. We aim to publish our full datasets after the study is completed so they can be re-used by other scientists.

Refinement



Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

In this project, we will use a rat model of high blood pressure. These rats have naturally occurring mutations and spontaneously develop hypertension. The pathology in these rats is like hypertension in humans, they respond similarly to clinical interventions (e.g. anti-hypertensive drugs), and drug-targets developed in this model have demonstrated a similar role in the pathology as in humans. In approximately one third of animals, we expect to use ex vivo techniques to measure progression of hypertensive heart disease. In the remaining, we expect to use mostly methods that allow measurements of cardiac function in conscious freely moving animals. These methods include the latest refinements available and are not expected to cause prolonged pain, suffering or lasting harm.

Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Because rodents are the lowest order in which blood pressure control is like humans. Further, control of blood pressure and cardiac function is significantly affected by general anaesthesia, therefore whenever possible, and welfare concerns can be addressed, physiological measurements must be performed in conscious animals.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

To minimize welfare impact, all recovery surgical procedures will be aseptic. Animals may experience transitory discomfort or pain as they recover; thus, they will be given painkillers and post-operative care just like people in the hospital. All animals undergoing surgery are expected to recover quickly. In the unlikely event of post-operative complications, adverse reactions, or if any animal fails to recover and exhibit signs of pain, distress or ill health, testing will be discontinued, and animals will be humanely killed. After recovery, animals will be free of pain and able to exhibit all their normal behaviours. Animals will typically be housed in social groups, but some experiments may require single housing for a limited amount of time. In consequence, animals may experience mild transient anxiety. All animals will have enriched environments and will be handled frequently to reduce boredom and anxiety. Whenever possible, medicines will be administered via non-invasive methods. However, some animals may experience mild transitory discomfort from receiving an injection. If repeat drug injections or blood sampling are required, the routes and volumes will be suitable for their age and size and will be such that animals fully recover between interventions and will not suffer more than transient pain and distress and no lasting harm and there will be no cumulative adverse effect. As stress and anxiety affect control of blood pressure, all animals will be handled using non-stressful handling techniques. They will also be allowed to adapt to experimental conditions in advance of experiments to reduce stress and may be given food rewards.



What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

<https://nc3rs.org.uk/>

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How will you ensure you continue to use the most refined methods during the lifetime of this project?

We will regularly consult the NC3Rs website and published literature for latest advances in 3Rs. Advances will be discussed with NVS and will be implemented if they are deemed feasible, sufficiently validated, and do not compromise the robustness and the comparative value of the designed protocols and ongoing studies.

Explain the choice of species and the related life stages

In this project, we will use a rat model of high blood pressure. These rats have naturally occurring mutations and spontaneously develop hypertension. The pathology in these rats is like hypertension in humans, they respond similarly to clinical interventions (e.g. anti-hypertensive drugs), and drug-targets developed in this model have demonstrated a similar role in the pathology as in humans.





187. Understanding the role of translation elongation factors in neurological 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

Epilepsy, Neurodevelopment, Neurodegeneration, Genetically altered, Mouse

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 broad aims of this project are two-fold, both relating to genes that encode proteins that are in turn responsible for making all other proteins in cells. Firstly, we want to understand why mutations in one of these genes cause epilepsy, intellectual disability and severe developmental delay, and ultimately to find and test new treatments. Secondly, we want to understand why nerve, muscle and heart cells undergo a highly conserved switch during early postnatal development between two almost identical versions of a gene responsible for making new proteins in these 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?

This work is important because the disorder that results directly from mutations in this gene is a devastating childhood condition. In addition, the gene we are studying is closely



involved in learning and memory and the processes that lead to neurodegeneration as well as disorders of the heart like cardiomyopathy.

What outputs do you think you will see at the end of this project?

By the end of this project we expect to have a range of mouse models of neurodevelopmental disorders caused by mutations in eEF1A2 and an understanding of how the different mutations work. Putting our mouse results together with clinical data on families we will have robust descriptions of the effects of the mutations and an understanding of the long term effects of the mutations on neuronal function. We will understand the functional differences between eEF1A1 and eEF1A2 and whether this can be exploited as a therapeutic strategy (in other words, can we upregulate eEF1A1 in nerve cells and use this to compensate for mutations in eEF1A2). All of these results will be written up as open access publications and publicised through our website and other social media, in consultation with the local communications team/press office.

Who or what will benefit from these outputs, and how?

The immediate beneficiaries will be the academic community, particularly medical researchers, who will have access to novel, well characterised lines of mice modelling clinically important and other mutations in eEF1A2. The academic community will also benefit from the publications that result from our work and the mechanistic insights we obtain.

The ultimate beneficiaries will be patients and their families affected by neurodevelopmental disorders resulting from mutations in eEF1A2. We are in a strong position to engage with this group because I set up and maintain a website largely aimed at the families of newly diagnosed children. We recently carried out a survey of eEF1A2 families to ask about their priorities for research, and the responses showed an even split between parents wanting gene therapy and those preferring other forms of medication.

How will you look to maximise the outputs of this work?

The mouse lines will be invaluable for any groups wanting to test therapies, whether genetic or drug based. In the past we distributed our antibodies to well over 50 separate labs on a no-strings basis, so we are confident that we can continue to engage with colleagues and collaborators on an active basis. Publication of the results will enable all beneficiaries to access our results and request reagents generated through this grant. The results will be disseminated through the normal channels of presentations at scientific conferences and publications both as preprints and then in the peerreviewed literature. Data will be deposited in appropriate open access databases. The University Press Office has extensive experience of generating press interest in publications and we will collaborate with them for any papers published.

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 although the gene we work on is active in cells grown in the lab, it is impossible to study because these cells also have another form of the same gene that swamps any effects of changing the gene we need to study. We also need to look at the effects in a whole animal because the human disorders we are modelling exert their main effects on behaviour, strength and learning ability. We need to look at animals from soon after birth until old age again because the human disorders onset soon after birth but can have changing effects on the body through to adulthood.

Typically, what will be done to an animal used in your project?

Animals will typically be born with a specific genetic defect that has a minimal effect when present in one copy but which, when present in two copies, result in neurological problems. In these cases the mice will be killed by schedule 1 killing while still moderately affected (failure to gain weight, more restricted patterns of movement). The mice with one copy of the genetic defect will usually be analysed by observation and weighing followed by a variety of motor tasks and behavioural assessment (not involving pain or food deprivation). In some cases, the mice will have a small wireless chip injected, of the sort used to tag dogs, in order to detect their movements in a cage of group housed animals.

What are the expected impacts and/or adverse effects for the animals during your project?

The main source of adverse effects will be in the homozygous mutant animals (i.e. those with two copies of the abnormal gene). They are likely to fail to gain weight after weaning and to move less freely around the cage but will not be kept any longer than 23 days or after 20% loss of body weight so these adverse effects will be transient. There is no evidence from human studies that these conditions are associated with 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)?

Most animals will be mildly affected. A small proportion, probably <20%, will be moderate.

What will happen to animals at the end of this project?

- 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 all experiments concerning the gene we work on, eEF1A2, we have to study animals rather than cultured cells. This is because the switch from eEF1A1 to eEF1A2 occurs in terminally differentiated cells, that is, cells that have irreversibly turned into muscle or nerve cells. In every cultured cell line (i.e. cells that can be grown in the lab) we have



examined, the cells express eEF1A1 at high levels (10-100 fold higher than eEF1A2 even in highly differentiated neuronal cell lines). This means it is very difficult to detect any effects of changes in eEF1A2 as they are obscured by the compensating presence of eEF1A1. We use biochemical techniques where possible to understand the consequences of the mutations that cause epilepsy and intellectual disability, but any effects on memory and behaviour can't adequately be studied without an animal model.

Which non-animal alternatives did you consider for use in this project?

Cell lines and zebrafish embryos.

Why were they not suitable?

Cell lines were not suitable for the reasons stated above. We removed eEF1A2 from zebrafish but this has no apparent effect as zebrafish have three other genes that compensate. This meant we could not get useful information from zebrafish embryos.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any.

These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate is based on numbers used in similar, closely related projects over the course of my previous PPL. Power calculations will be carried out for specific tests/lines after pilot studies are completed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use online tools and seek advice from local statisticians on an ongoing basis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies will be used for each new line and each test. We keep breeding lines to a minimum and aim to use all animals where possible (including using heterozygotes which have had minimal nonprocedural phenotyping for breeding purposes). We make a tissue archive for each new genotype which can be used within our group or shared with others.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.



Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice which we will manipulate genetically using a gene editing technique that is highly efficient. There is no valid alternative to any of the techniques we are using, all of which have been chosen to reflect the human conditions we study but with as little stress as possible to the animals.

Why can't you use animals that are less sentient?

The gene we study is not switched on properly until well after birth and can't use terminal anaesthesia for behavioural and observational testing. Less sentient species do not have the gene, which is restricted to vertebrates.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals with two copies of the mutant gene will be monitored daily from weaning and will be humanely killed before their condition deteriorates beyond moderate severity. None of the procedures are thought to cause pain. Animals doing behavioural tests will be habituated to handling and put through a standard pipeline for tests, many of which will be carried out using the same equipment (maze, open field) adapted for multiple purposes.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the PREPARE guidelines from Norecopa and be mindful of the ARRIVE guidelines for reporting, since an awareness of ARRIVE also helps with experimental design.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I receive the NC3Rs newsletter and follow them on Twitter. Via my role on our local AWERB I receive and read regular updates from the ASC and Norecopa, for example, together with The Hub AWERB newsletter. I ensure that I pass on new developments to people in my lab (we started using tube handling as soon as we heard about it, for example).





188. Using *Xenopus* as a tool to support clinical genetic diagnosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

disease models, rare genetic diseases, gene editing, *Xenopus tropicalis*, behavioural analysis

Animal types	Life stages
<i>Xenopus tropicalis</i>	adult, embryo, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To support the diagnosis of rare genetic diseases we will recreate the DNA changes identified in human genetic disease sufferers in tadpoles and frogs. We will compare the effect of the genetic change on tadpoles with the symptoms of the disease to discover whether the genetic change causes the disease. If opportunities arise we will use tadpoles containing the gene variants to screen potential therapies for elements of the human disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?



The availability of high throughput, next generation DNA sequencing revolutionised our understanding and mitigation of human genetic diseases by enabling clinical research to identify causal variants within a gene in both congenital and later onset clinical disease. DNA sequencing was undertaken at a very large scale in the UK by the 100 000 genomes project that was launched in 2012 and this programme is currently being greatly expanded. A challenge in making use of this sequence data is however sensitive interpretation of variants of unknown significance (VUS).

VUS are changes to genes in which the certainty of the link between the gene(s) identified and the disease they cause does not meet that required for clinical decision making. It has been a surprise that in around half of the genetic disease sufferers sequenced it is not obvious which altered gene is responsible. There is therefore a major bottleneck in diagnosis for clinicians and patients, making commonplace what is known as a “diagnostic odyssey” for patients with these diseases. On average it takes 4 years for diagnosis and this is important; overall 30% of children with rare diseases survive for less than 5 years but this improves to 80% when they have diagnoses. Having no diagnosis also affects the patient’s family, with 83% reporting the need for extra support for their mental health. In order to assess VUS functionally, a rapid way of testing whether the variant causes a disease is required. While primary cell cultures, organoids and biochemical studies are informative in some cases, these cannot reflect the complex interactions that occur within tissues, organs and whole organisms. Although mammalian models can be used, the ethical and financial cost is high so alternatives are sought. Of these, *Xenopus* is most highly related to mammals and alone shares its genome architecture with humans. Our data show that *Xenopus* is an effective model in which to make and test whether a VUS causes a human disease. As the programme develops we will start to investigate screening of potential therapeutic agents for the human disease using the genetically altered tadpoles.

What outputs do you think you will see at the end of this project?

This programme will determine how useful re-creating human genetic variations in *Xenopus* can be for the diagnosis of rare genetic diseases. This will be achieved using gene-editing techniques. By working in collaboration with clinicians treating the patients with the genetic variations we re-create we will understand how useful these data are to inform their decisions and interventions.

A side benefit of this work for other scientists is that new, developmentally important genes are likely to be discovered.

In the long term, *Xenopus* embryos and larvae are excellent for high throughput screening of small molecules. By developing the gene-edited tadpoles containing the human disease gene variants as detailed above we will have made screening tools for the discovery of molecules that can treat some aspects of the disease and this is now an objective of the programme.

Who or what will benefit from these outputs, and how?

This knowledge will initially benefit clinicians working with patients suffering from rare genetic diseases and researchers using *Xenopus* by determining whether gene editing in *Xenopus* can be used successfully for the diagnosis of rare genetic diseases.

If the programme is successful then it will become focussed on clinical support and so patients suffering from rare genetic diseases will benefit.



Tadpoles may be used to evaluate potential therapies. For example, tadpoles carrying the VUS may be used for screening libraries of small molecules to test whether any alleviate the symptoms of the genetic disease. This would benefit some patients and the pharmaceutical industry.

The identification of developmentally important genes will be of benefit to developmental and cell biologists studying basic embryology. They will be able to integrate the data about the function of these genes into the genetic regulatory networks that drive development. This will lead to a better understanding of developmental processes.

How will you look to maximise the outputs of this work?

Our collaboration with clinical partners and our network of >200 *Xenopus* laboratories that use our resources will enable us to maximise the outputs of this work. For clinicians, there are well established, web-based databases to which the proven connection between a VUS and disease can be added; any that we discover will be added there in a timely manner (as has already been the case for some of the experiments).

For developmental biology outputs, our regular contact with so many research groups will allow us to send the data and gene editing tools for any developmentally-important gene to the group(s) studying the relevant cell type or process immediately.

For unsuccessful approaches, Wellcome Trust funding allows us to publish negative results in Wellcome Open Research, which we will do.

Species and numbers of animals expected to be used

- *Xenopus laevis*: 0
- *Xenopus tropicalis*: 15600 tadpoles, 504 adults

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using frogs to test how helpful their tadpoles can be in helping clinical geneticists to answer whether a variation in the human genome is the cause of a rare genetic disease. *Xenopus* frogs are the animals of choice for this programme because they are similar to humans in terms of their genes and the structure of their genomes but produce large numbers of externally fertilised eggs and accessible, synchronously developing embryos which means that many of the ethical challenges around using mammals for such work are avoided. Our data show that variations in the human genome which cause genetic diseases almost always cause the same "disease" change when the variations are re-created in frogs. In general we can use embryos and tadpoles for these experiments because the changes to the animal caused by the gene variant are clearly detectable. The exception is alterations to bones, lungs of limbs which only appear at metamorphosis in the frog. As the project has progressed we have noted the need for heterozygous models to be bred for some diseases, this means keeping highly mosaic animals with a harmful mutation to adulthood and breeding from them, we have recently observed very rare instances of harm in these animals and now include this procedure on the licence. Since



the alternative model for such work would be mice we remain confident that *Xenopus* are the best choice of model for this work.

Typically, what will be done to an animal used in your project?

For adult animals which are used to produce embryos for these experiments the females have an injection of hormone and the next day are gently massaged to stimulate them to lay eggs, since this procedure causes very little discomfort to the animal it can be repeated 15 times at 90 day intervals.

The eggs are fertilised using frozen sperm from male frogs that have been killed humanely. One type of genetic manipulation needed to re-create the human disease gene variations in frogs requires oocytes, these are removed from the ovary of a frog under anaesthetic. High quality oocytes for these experiments are not found in all frogs, so frogs that produce high quality oocytes will be re-used up to 4 times. We will also test whether microinjection into an ovary lobe outside the frog whilst it is under anaesthetic can be used to produce this type of human disease variant in frogs.

The genetically-altered tadpoles produced from these embryos and oocytes normally have the "disease" phenotype found in the human with the gene variant that they carry. In addition to the adverse effect of this, they may be anaesthetised (up to 4 times) or treated with a neuromuscular blocking agent to allow them to be observed, they may also be treated with a chemical that causes seizures or undergo behavioural testing by swimming in coloured or unusually shaped tanks while being filmed. These genetically altered tadpoles may be grown up to adulthood and bred from.

What are the expected impacts and/or adverse effects for the animals during your project?

For the adult animals that produce eggs for embryos, long experience shows that the adverse impact is very limited. Fewer than 1 in 500 animals shows any sign of egg retention or infection at the injection site. If they have not recovered after 48 hours of treatment these animals are killed humanely, limiting the period of suffering. For oocyte removal from *X. tropicalis* the situation is much less clear since it is only being performed in two establishments in the US, direct contact with them reports that this is much the same as when the same operation was done routinely on *X. laevis* and that infection or failure to heal has not yet occurred in the limited number of animals used. Were either of these to occur then after 48 hours of treatment these animals would be killed humanely, limiting the period of suffering.

For the affected tadpoles 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 are normally killed humanely after less than 10 days. Adults carrying the harmful gene mutation are made as highly mosaic animals or bred as heterozygotes, in very rare cases we believe these can still have the disease. Any showing harmful phenotypes will be humanely 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)?



Female Xenopus for egg production: Mild (1.2%)
Male Xenopus for sperm production: Mild (0.3%)
Female Xenopus for oocyte production: Moderate (0.4%)
Genetically altered tadpoles: Mild (97.1%)
Genetically altered adults: moderate (1%)

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are needed for this work since the effect of VUS on different systems within the organism normally needs to be tested. The very nature of the work means that the experiments will be assessing the specificity of the VUS, an essential part of which is determining whether it affects systems that do not correspond with those affected in the patient.

Which non-animal alternatives did you consider for use in this project?

For the testing of VUS with defined targets cell types for which there are well-established cell and organoid culture-based analyses, these are already undertaken in appropriate circumstances as part of the pipelines used by the group with which we collaborate.

Why were they not suitable?

Only in circumstances when cell and organoid culture-based analyses are uninformative will animal research take place.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These are based on the numbers needed for the preliminary experiments, which have effectively been pilot studies for this investigation.

Tadpoles: For each gene we are studying we will keep 50 tadpoles beyond feeding stage in each experiment, this allows for the natural death rate of tadpoles and to have 30 animals for phenotype analysis. We perform 3 biological replicates of each experiment. For the actual test experiments we will re-create 50 VUS, each requiring 150 tadpoles;



7500 tadpoles in total. In addition to the tadpoles used for these "diagnostics" experiments there will be some used for the method development, these numbers are much more difficult to predict but at present we are using around 90 per week for a similar process. Making the assumption that half of the duration of the project will involve method development this would be 120 weeks using up to 100 tadpoles; 12000 tadpoles in total.

Adult animals: We will share embryos between this programme and others since so many embryos are produced by each female that many experiments can be completed using them. We will allocate 1 female each week for 48 weeks of the year to producing embryos for this programme; 240 females. Depending on the breeding state of the male frogs it may be necessary to prime them with PMSG prior to freezing their sperm for *in vitro* fertilisation or ICSI, a single male each month will be allocated to this project since frozen sperm from a single male can be used across 16 experiments; 60 males. For oocyte removal with recovery, we will use two frogs every three weeks and re-use them four times, totalling 64 *X. tropicalis*. We expect to need up to 10 adult animals for any VUS to propagate lines.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We were able to use data from our earlier experiments to understand how many animals would be required to produce robust data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

There are two main approaches that we use to keep the number of animals used in the project as low as possible; first we use frozen sperm to produce the embryos. Since we are using *X. tropicalis* we can certainly fertilise 16-fold more clutches of eggs with frozen than with fresh sperm, this clearly decreases the number of male frogs used 16-fold. Secondly we share eggs across the four *Xenopus* using research groups based here. We hold a weekly meeting of users to ensure the minimum number of animals is used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use *Xenopus* because:

Large numbers of *Xenopus* embryos are produced externally and obtaining them requires only a mild procedure (injection of HCG into the female).

We will use tadpoles prior to metamorphosis, when the brain is poorly developed and lacks the region that, at least in man, generates the distress associated with pain. The tadpoles are largely transparent, allowing simple observation of many of the phenotypes produced without invasive methods.



We have developed efficient phenotyping protocols which maximise that data produced from a single mating.

High throughput screening of potential therapeutics is well established in *Xenopus* due to their large clutches and rapid development.

Why can't you use animals that are less sentient?

The frog's genome is very similar in structure and detail to the human one and more than 80% of the currently-identified human genetic disease genes are found in it. To use a less sentient animal in which the genome is sufficiently well annotated for these studies means using zebrafish or *Drosophila*. The latter, in many cases, lacks sufficient protein identity to humans to make it possible to re-create VUS. The structure of the zebrafish genome is very different from that of humans/frogs. Currently it is also not possible to produce embryos from zebrafish oocytes, limiting the ability to re-create VUS that require this methodology.

In terms of developmental stage, we use the earliest possible for all experiments but have to judge this on a case-by-case basis depending on the cell type or organ affected by introducing the VUS.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The whole of this programme can be considered a refinement since it uses a less sentient animal than mice, which are currently used for this type of work.

The main refinement that we aim to generate in this programme is the ICSI of *X. tropicalis* oocytes; at present these oocytes are able to be fertilised only after placing them into the ovary of a laying *X. laevis* female. Although we can do ICSI successfully in *X. laevis*, it has proven very difficult to get *X. tropicalis* embryos made this way through neurula stages, although we have not given up. As an alternative we propose to microinject oocytes within an ovary lobe outside an anaesthetised frog with the gene editing machinery, replacing it, allowing the frog to recover and then lay the GA eggs three days later. Either ICSI or the proposed approach will greatly speed up production of non-mosaic heterozygous models and prevent the growth and breeding of mosaic adults, hence it is a refinement.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The guidance available is mainly around animal husbandry rather than experimental procedures but we do both follow and have contributed in an editorial capacity to the UFAW handbook, Sherril Green's excellent "The Laboratory *Xenopus*" (which is currently being updated) and the RSPCA welfare guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Fortunately the place of work is very well connected with the community of researchers using *Xenopus* and so staff attend all of the meetings about the use of this model animal, ensuring we know about any advances even prior to publication. We also generate and propagate 3Rs advances, for example the sperm freezing and ICSI used in this programme were developed into routine procedures by us.





189. Utilisation of a seeding model with human α synuclein to induce Parkinson’s Disease

Project duration

1 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

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 main objective of this project is to understand the mechanisms underlying the development and progression of Parkinson’s disease using a novel animal model.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The main benefit of this project will be to enhance our understanding of the brain processes involved in the emergence and progression of the disease which could facilitate the development of new therapies/improved medication for patients.

What outputs do you think you will see at the end of this project?



This programme of work will further our understanding of disease progression of Parkinson's disease by studying changes in both neural and behavioural processes. The key aspect of the research is the development of a novel more refined experimental model for Parkinson's Disease which closely mimics the symptoms and disease progression observed in patients and will therefore be more translationally relevant.

Who or what will benefit from these outputs, and how?

The data generated from this research project will guide and inform both our research and could also have benefits for the wider research community. In the long-term it is envisaged that this model will not only facilitate a better understanding of the disease, but it could also be utilised in pre-clinical testing and the potential development and screening of new treatments/compounds by pharmaceutical companies and therefore in the longer term it will benefit patients along with their carers/families.

The present research is vital with the increased life expectancy and a larger proportion of the population aged over 65 years, which in turn has resulted in an increased prevalence of individuals with age-related diseases including Parkinson's disease.

This present application is for a bridging licence for an animal cohort that has already been surgically inoculated with seeding material and is now in the process of disease manifestation and progression (all animals duly recovered and healthy) awaiting their final endpoint assessment.

How will you look to maximise the outputs of this work?

The findings and data from this project where possible will be published in peer reviewed journals and will also be shared with the wider research community via presentations at both national and international scientific conferences.

Species and numbers of animals expected to be used

- Mice: 46

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 to be used in this project are adult mice who have previously undergone surgery on our current licence, and all these animals are now fully recovered and healthy. These mice represent a novel model for Parkinson's disease as they are able to mimic the development and progression of the disease similar to what is observed in human patients.

Typically, what will be done to an animal used in your project?

Animals will undergo behavioural assessment utilising various tests of olfaction and activity related behaviour which are symptoms associated with the progression of Parkinson's disease.



What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected with all animals healthy and not displaying any adverse effects following surgery.

The behavioural tests that will be utilised during the course of this project are themselves not expected to induce any adverse effects on the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The present project is classified as moderate severity as all of the animals have previously undergone surgery however, the behavioural tests are seen to be only mild in severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to study and understand complex behaviours including olfaction and activity and related brain function we need to use the whole animal.

Which non-animal alternatives did you consider for use in this project?

Unfortunately no suitable alternatives are available for this project. However, tissue will be harvested at the end of the study and extensively analysed.

Why were they not suitable?

The behavioural assessments which will be utilised in this project can only be performed on the whole animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?



The number of animals that will be used for this project are those that have previously undergone surgery on our previous licence and are awaiting behavioural assessment. This number was based on our previous experience and data using a seeding model.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Based on our experience and the results from a previous study we are using the minimum number of animals needed to attain statistically robust results. Given the uniqueness of this study, there was no power projection analyses. We will also implement a battery of behavioural tests to prevent the need for using multiple animals for the different tests.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Tissue will be harvested from all of the animals at the end of the study and will be analysed allowing us to attain the maximum amount of data from a given 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 mice that will be used in this project have previously undergone aseptic surgery and been inoculated with alpha-synuclein and are all healthy.

During this present project they will undergo behavioural assessments to determine effects on olfactory ability and activity. These tests are not expected to cause any suffering or distress to the animals.

Why can't you use animals that are less sentient?

The animals to be tested in this project have previously undergone surgery and behavioural testing will be performed at 8 - 9 months post surgery to allow appropriate spread of the alpha synuclein protein within the brain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The development of new animal models that more truthfully mimic the symptoms seen in patients will allow us to understand the diseases better.

We have introduced standard operating procedures to regulate and standardise all methods and techniques used in our research. To try and minimise the harm and stress that the animals undergo they will be group-housed as much as possible and all researchers will be fully trained and assessed for competency.



For behavioural testing we will utilise advanced video observation tools and novel computational analysis tools/software to facilitate a more sensitive and effective profiling of an animals behaviour.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

At all stages of experimental design, we will follow ARRIVE 2019 guidelines with experimental blinding and randomisation in order to avoid any bias. We are also in the process of implementing a novel 'Data Quality System'

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continuously strive to apply the 3Rs to our work. In order to do this we will follow the literature, NC3Rs, CRACKIT and Norecopa.



190. Vaccination against COVID19 and related coronaviruses

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

No answer provided

Animal types	Life stages
Mice	adult, aged, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims at providing promising vaccine candidates for the COVID19 pandemic. In full recognition of the great number and scope of already ongoing COVID19 vaccine initiatives, this project wishes to address the very real possibility that existing vaccine candidates may prove ineffective and/or unsafe and to provide an alternative which may bypass limitations of other candidate vaccines.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The COVID19 pandemic cannot be managed in the long run without a workable vaccine. Hence, vaccine development has been identified globally as a priority to combat the



condition. Other candidate vaccines, currently entering phase I clinical development, for the most part target a certain element of the coronavirus causing COVID19 which would block the virus from entering host cells. However, similar strategies, attempted for over a decade for the closely related SARS coronavirus, have shown that - in addition to being of uncertain efficacy - vaccines may actually cause worsening of pneumonia in virus-infected patients. The current project therefore pursues an alternative vaccine design specifically aimed at bypassing these limitations. In the event that on-going vaccine trials prove unsuccessful, such alternative designs will be urgently needed.

What outputs do you think you will see at the end of this project?

The output of this work will be the demonstration that COVID19 candidate vaccines elicit neutralizing antibodies and are suitable for testing in virus-infection studies.

Who or what will benefit from these outputs, and how?

The most promising vaccine candidates will be transferred to collaborating institutions equipped to conduct in-vivo COVID19 infection studies in relevant animal models. This research will not only demonstrate whether the vaccines can prevent infection but also, crucially, whether they avoid worsening of infection-related pneumonia which has been identified as grave risk for other coronavirus vaccines.

How will you look to maximise the outputs of this work?

We have already set up collaborations with long-standing partner groups well equipped to conduct flanking and downstream studies for vaccine characterization, as well as in vivo protective capacity.

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 use of vaccines in humans requires prior demonstration that they can achieve their intended activity of making the body immune against a pathogen. This can be done with the least number of animals initially with mice which are "inbred" . This means they are very similar in their immune response. This reduces the number of animals that need to be used. In addition, the response to vaccines differs between young and old. For that reason it is necessary to test the vaccine response in both young and old mice.

Typically, what will be done to an animal used in your project?

Typically, mice will be housed in group colonies with enhanced environment, in compliance with current UK guidelines. They will have a blood sampling before vaccination and then be vaccinated , followed by further blood test to see if the vaccine generated sufficient antibodies against the pathogen. As with humans, vaccinations may be repeated (so-called 'booster' vaccinations) and be applied through injection into the skin or as nasal spray.



What are the expected impacts and/or adverse effects for the animals during your project?

(As with humans), it is assumed that mice experience subcutaneous injection, dosing with nasal spray, and phlebotomy via tail vein as unpleasant. However, the impact of this is brief and mild discomfort and appear not to cause lasting pain 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)?

The severity of suffering is 'mild' as detailed above: it is incurred by vaccine given as skin-injection or nasal spray, as well as by venous blood sampling. This applies to all mice studied.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Regulators such as MHRA, FDA, and EMA require proof-of-principle for any novel candidate treatments, including vaccines, to be carried out in suitable animal models, such as rodents, before moving into clinical phase testing.

In addition to evidence of efficacy, pre-clinical testing is required to obtain basic information regarding the mode of action of new candidate treatments. In the present case this relates to immunodynamic as well as immunokinetic aspects of novel vaccines to be studied. There are no cell culture models that could substitute for this requirement.

However, we will nevertheless incorporate elements of replacement. For example, wherever possible, we will utilise serum ascertained from mice dosed with vaccines and use the serum - contained vaccine-triggered antibodies in in-vitro bioassays in cell culture to quantify the effect of vaccines. This will minimise the frequency with which animals will have to be subjected to any vaccination at all.

Which non-animal alternatives did you consider for use in this project?

None are applicable for vaccination studies

Why were they not suitable?

The response to vaccination requires the interplay of many organs, tissues, and cells in the whole body and cannot be emulated by specific cells or other test tube 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?

We have extensive experience with the number of animals per treatment arm for each vaccine candidate. For each study we also need to take into consideration studies addressing important dosing variables including: route of administration, amount of vaccine for each dose, use of added adjuvant, use of pre-vaccination with tetanus, studying the response in old vs young mice.

In addition, it is possible that COVID19 vaccine responses differ in risk groups, such as patients with diabetes, asthma, or high blood pressure. If so, this would need to be studied in animals.

Therefore, we expect that the actual number required to validate a vaccine candidate suitable for clinical development to be much lower than the number indicated but, equally, all the above detailed consideration make it at least possible that up to the above number of mice may need to be used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

First, we will only conduct experiments with candidate vaccines that have a high likelihood of achieving good efficacy and causing no or minimal toxicity based on current knowledge. Thus, we will specifically only use one single strain of mice for the initial screening of vaccine prototypes.

Secondly, based on our extensive previous experience with VLP-type vaccines we have a good understanding of the required minimal group sizes of animals to achieve signals not confounded by experimental variability. This will also contribute to reduction in required number of animals.

Third, to further reduce animal numbers, we will only perform alternative-route vaccination (intranasal) as and when a vaccine prototype has been shown to generate any anti-body response by the default (subcutaneous) route.

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 adopt an iterative approach such that, as and when we observe that the variability of immune responses is lower than expected, we will proceed to reduce group sizes.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the



mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 routes of vaccination studied here, subcutaneous injection and intranasal spray, are the routes to be used in humans for COVID19 vaccination with these types of vaccines. As such they represent the only adequate model.

The use of specifically inbred strains C57BL6/J and Balb/c reduces the number of animals required by taking advantage of highly standardized immune response. In addition, these two strains between them serve as very well characterised models of general types of immune responses observed in humans, thereby covering a wide range of vaccine responses with only two models.

Why can't you use animals that are less sentient?

There are currently no established less sentient or terminally anaesthetised animal models allowing vaccine studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Inbred C57BL6/J and Balb/c mice have been used widely for initial vaccine testing and found to be of good general predictability for antibody-titre formation. The ultimate (current) model of COVID19 disease is Golden Syrian hamster.

However, performing immunogenicity studies in mice represents a significant refinement as it minimizes any live virus-infection studies to the testing of vaccine prototypes, and spray-aerosol antiCOVID19 drugs, to the characterization of promising candidates.

In order to minimize animal suffering, we will limit the number of blood sampling to the absolute minimum required to obtain a definite answer to the scientific question at hand (in particular: the time course of anti-COVID19 antibody development). We will continuously review and adapt usage of animal numbers to incorporate emerging observations of treatment effect size and variability in power calculations so as to possibly even further minimize the number of animals used.

For other routes of drug application we will use short term anaesthesia to minimize animal distress and pain as appropriate for each route.

The following principles will be adhered to in order to minimise hazard and toxicity for affected animals:

all vaccine batches will have been tested for levels of endotoxin and principle sterility prior to initial use.

all vaccines will have been formulated with reagents, buffers and adjuvants not known to incur toxicity to mammalian organisms at the concentrations employed.

A further refinement is the use of light anaesthesia for intranasal vaccination to further reduce animal discomfort.



A final method of refinement is the use of terminal anaesthesia for killing. This allows for sampling of increased amounts of serum in each animal and therefore increases the ability to detect changes in blood constituents of low abundance (for example basophil counts) without having to pool sera from several animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidelines available at <https://www.nc3rs.org.uk/guidelines>.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I continuously monitor and review emerging scientific literature on COVID19 related vaccine progress world-wide and am in close contact with collaborating scientists working on related research questions.



191. Validating computer models of mastication

Project duration

5 years 0 months

Project purpose

Basic research

Key words

feeding, dental implants, modelling, rabbit

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The goal of this research study is to demonstrate that computer models are capable of accurately predicting the internal forces generated and sustained by the muscles and bones of rabbits while they feed. This will be achieved by comparing the predictions from models to a new set of direct experimental measurements on rabbit muscles and bone. This will be pursued through the following specific objectives:

- collect in-vivo data on bone motion and muscle physiology on rabbit mastication
- collect in-situ/ in-vitro data on the physiology of rabbit masticatory muscles and the mechanical properties of hard tissues from the skull and mandible
- collect anatomical and image data on bone and muscle morphology in rabbits
- combine data from (1), (2) and (3) to build and validate computer models of rabbit mastication.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

The goal of this project is to demonstrate that computer simulation approaches can contribute significantly to reduction, replacement and refining of the use of animals in biomechanical research. Many experimental studies in animal biomechanics are highly invasive, causing pain and distress to the animals before they are euthanized. In theory, once a digital model has been created, computer simulation has the potential to



completely replace (or maximally reduce) the use of animals in that area of biomechanical research and/or medical device design. The anatomy and/or behaviour of a digital model can be manipulated or altered continuously without any harm or distress to a real animal. This can allow, for example: a model analysis to be extended to a different strain/breed of the same species (or a morphologically similar species) by digital modification of the anatomy/behaviour; elements of anatomy to be modified in multiple ways (e.g. removal of teeth/bone) to examine the consequences of different surgical approaches; and for implant devices to be digitally inserted into models to examine their mechanical impact and performance, all without the need for any experimentation on real animals.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

We will use approximately 45 male New Zealand White rabbits over the course of the next four years to generate the data required to build and thoroughly test our models. The maximum of 45 rabbits was decided on based on analysis of previously published data and a power analysis.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The likelihood of adverse reactions to the experimental interventions are low. There is potential for inappropriate anaesthetic depth that could result in moderate pain or death (less than 1% likely incidence). All animals will be killed by a Schedule 1 method at the end of the experiment.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

To validate a computer model requires a large amount of data about the anatomy and mechanics of feeding used by rabbits. This data does not exist for rabbits, or indeed any other experimental animal. Therefore a systematic anatomical and biomechanical investigation of rabbit feeding is required in which all the primary determinants and measures of feeding are measured from a small cohort of rabbits. Constructing models from medical imaging data of those same rabbits can then directly and immediately validate computer simulation. Only in this way can models be truly validated and their potential for achieving 3R's in future studies be demonstrated.

Reduction

Explain how you will assure the use of minimum numbers of 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 consulted to ensure the minimum number of animals is used

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Rabbits are the first-choice experimental animal for dental implant design studies because of their size and easy handling. According to international standards regarding species suitable for testing implants in bone, rabbits represent an important species. Although the rat is also a frequently used model, it is not really regarded as a suitable model for testing dental implants and bone remodelling due to significant differences in bone composition, healing, and anatomy to humans. Therefore because rabbits represent by far the most widely used species in this context we proposed to use them to demonstrate the capacity of validated computer modelling as a means of achieving replacement, reduction and refinement of animal use in future studies of dental surgeries and implant design. We have the facilities and expertise for housing this species. In this project we will use male New Zealand White rabbits because their large body size (~3kg) is more amenable to the surgical procedures and xray imaging than smaller breeds of rabbits.



192. Vertebrate models of ciliopathies.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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 aims mentioned in purpose (b)

Key words

Mouse, Zebrafish, Genetically altered, Surgery, Drug treatment

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The Cilia Disorders Laboratory aims to find changes in the DNA sequence of patients with disease, known as mutations.

To study these mutations we will:

Make these same mutations in mice and zebrafish, as appropriate;

Test new treatments using these mice and zebrafish.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

By finding new causes of disease we will help parents to understand their child's disease, and the work with animals will help us to understand what causes the disease and to find new treatments.

Species and numbers of animals expected to be used



What types and approximate numbers of animals will you use over the course of this project?

The period of time for this project licence is 5 years.

Mouse: We will use approximately 6000 mice during this project over 5 years.

Zebrafish: We will create or maintain approximately 4000 zebrafish over the 5 years of this project.

Rats: Where optimised protocols require us to extract rat serum we will do so and we anticipate to use up to 100 rats over 5 years. Where possible mouse serum will be purchased commercially.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Genetically-modified animals: several of the mutations that we will generate and breed could cause diseases at very early stages, before the animal is born (mice) and/or before it feeds independently (zebrafish). At these stages, the animals are not thought to suffer in any way and are not subject to specific Home Office restrictions, and we will not allow them to develop further than this.

Some animals may have mild or moderate problems which may sometimes cause mild or moderate discomfort later in life. This might include abnormal teeth, extra fingers or toes, or poor vision. Such animals are monitored closely to look for adverse signs (e.g. weight loss) but usually live a normal life.

Data acquisition, associated procedures and human end-points are all listed in detail in the project plan and for each protocol. To summarise three general procedures and associated adverse effects are relevant:

Breeding and maintenance of genetically modified mice and zebrafish - it is possible that certain phenotypes will cause adverse effects. In zebrafish, this is the scoliosis which can affect swimming and competitive feeding. This is a moderate adverse effect and accommodate by close monitoring of animals, placing groups of affected individuals in separate tanks and sacrificing severely affected animals (e.g. inability to swim or feed). For mice, most phenotypes are embryonic. The postnatal phenotypes that cause adverse effects include renal cysts, partial blindness, obesity and craniosynostosis. None of these phenotypes cause severe adverse effects within the time-periods concerned. The craniofacial defects can be associated with malocclusion and thus tooth trimming is required to permit feeding on a wet diet.

Injectable drug - the compounds proposed to be injected include tamoxifen and FDA-approved drugs. These compounds will be used at standard dosing, usually associated with no side-effects. In a minority of cases, it is possible that some compounds will cause



illness through immunocompromisation or other unknown mechanisms, and these animals are monitored carefully for adverse changes in behaviour and appearance and removed from drug treatment and/or sacrificed where necessary. Similarly, injection sites could become infected causing severe illness. Such animals will be treated following the advice of the NVS or sacrificed as appropriate.

Imaging and anaesthesia - in a small number of cases, animals may be anaesthetised using the stated routes of administration and doses for imaging (X-ray). It is expected that animals will recover from this without harm. It is extremely unlikely, but possible that animals will be injured when constrained for imaging - highly unlikely injuries include broken limbs or internal bleeding. Just animals will not be recovered from anaesthesia or sacrificed at a later date if complications are noticed after recovery from anaesthesia. Surgery - Severe adverse effects could result from surgery and general anaesthesia where treatment of craniosynostosis is studied. Infections could result or animals could become seriously unwell as a result of the general trauma. While unlikely, such animals will be sacrificed.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

In order to understand human birth defects and other diseases later in life, it is necessary to study organs and tissues within the context of a whole organism. This is because the affected tissues are complex and 3-dimensional and this cannot be studied simply by studying cells grown in a dish in the laboratory, for example, and sometimes diseases later in life involve interactions between multiple organs. Equally, it is essential to test new treatments in animals because drugs are processed by the body before reaching the diseased tissue.

Where possible, we do replace animals with cell culture systems, including use of immortalised cell lines, or primary cells (e.g. grown from a small piece of skin) from which stem cells can be grown and then made into organs such as kidneys, eyes etc.

Reduction

Explain how you will assure the use of minimum numbers of animals.

We use the minimum number of animals possible by designing our experiments carefully. In pilot experiments, we test new treatments in the test tube, without using animals, to provide initial justification to go on to perform experiments in animals. We will also perform initial experiments on small numbers of animals to check for adverse effects, and to determine the most effective experimental design to reduce overall animal numbers but also to reduce severity (i.e. to optimise drug treatment doses or avoid adverse effects of mutations in large numbers of animals).

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.



For many of the diseases we study, patients carry specific mutations that have milder effects than complete gene knockouts – mutations that are predicted to wipe-out gene function completely.

For treatments, pilot experiments will be performed to identify the lowest effective dose, for further testing in larger animal populations. This will reduce adverse effects in large numbers of animals.



193. White matter injury mechanisms in diseases of the nervous system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

White matter, injury, nervous system, stroke, multiple sclerosis

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

About 50% of our brain is made up of white matter, which is damaged in stroke, dementia, cerebral palsy and multiple sclerosis. White matter is made up of axons, glial cells and myelin. Axons transmit information around the nervous system and glial cells support this process. Myelin is an insulating layer that surrounds axons which is damaged ("demyelination") in diseases like multiple sclerosis. Studying injury to white matter requires the capacity to identify the different type of glial cell reliably and this project will use transgenic mice with fluorescent proteins located within glial cells to overcome this hurdle. Various models of injury will be used and this license covers the use of a transgenic mouse model of dementia and a mouse model of demyelination. The objective is to explore how different cells within white matter suffer damage and to test the idea that drugs targeted to specific receptor proteins may be protective.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

We aim to better understand how injury occurs in white matter in a number of diseases and to progress work toward drugs that protect the tissue. In particular we will investigate



the role of neurotransmitter receptors and drugs that block them. Our work will move toward the development of effective interventions against a variety of neurological disorders. White matter injury is critical in numerous neuropathologies, including stroke, dementia, cerebral palsy and multiple sclerosis. Stroke is the third most significant disorder in Western society, cerebral palsy is the most common neuropathology of immaturity, for example. The strategy is to test very low combined concentrations of drugs which are likely to have few or zero side-effects and may therefore be suitable to protect the brain in patients at risk or those suffering chronic progressive conditions such as some forms of multiple sclerosis. The project will take 5 years and we then hope to develop our findings for clinical trials.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

This project will last for 5 years. During this time, we plan to use ~1000 mice per year. For the genetically modified mice, about 50% of these will have no abnormal phenotype and will be used as controls and the remaining 50% will have a mild or moderate phenotype. ~60 mice/year will be used in a model relevant to demyelination and ~40/year will be given drugs in the diet. In both cases the severity limit is moderate.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

The transgenic animals are bred and killed humanely, with tissue used ex-vivo. Some animals will be treated with drugs and closely monitored. The transgenic strains are already well characterised and no health concerns above “moderate” have been identified. Animals fed cuprizone to produce demyelination will be kept on the diet for up to 6 weeks and are likely to lose up to 10% of their body weight with a moderate severity. Animals treated with drugs to block neurotransmitter receptors may experience moderate neurological symptoms. Animals will be monitored daily by staff with extensive training and expertise in animal care.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

We will use mice for these studies as there are a large number of tools (in particular, transgenic models available in this species. Rodents also have a nervous system that contains white matter, which we wish to study. Lower organisms such as flies, fish and worms have no white matter. A significant amount of the work we do relies upon using cell culture systems, but cell injury in the nervous system involves a complex interaction between many different cells types and therefore cannot be accurately modelled in vitro. We can get useful information from the cell culture experiments but the validation of these results needs to be performed using animal models such as the transgenic mouse models employed in this project.



Reduction

Explain how you will assure the use of minimum numbers of animals.

The number of experiments in our work is kept to a minimum. Small numbers of experiments will be conducted first and if these are successful with acceptable levels of animal welfare the numbers of experiments will then be increased. The total number of experiments required will be determined by a statistical analysis. Precise numbers of animals used for each experiment will depend upon the scatter of the data and the appropriate statistical test(s) used to analyse the data; advice will be taken from medical statisticians within the University where appropriate. Strict training and supervision of those post-doctoral workers and PhD students performing the work will limit the number of experiments (and therefore animals) rejected from analysis for technical reasons (failed experiments). It is important to recognize that using transgenic mice to identify cell type is far more efficient than methods, such as post-mortem cell staining, that are applied to wild-type animals. The breeding and utilization of these models will therefore represent a significant reduction in the total number of animals required to reach meaningful conclusion. Good experimental practice will be used throughout. For example, where possible the person conducting the experiment will not know if they are testing a control or a test condition (blinding), and experiments will be done in a randomised order.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Much of the work studying myelin injury in this project will use acute in vitro models, some of which are novel. Use of these models will represent a refinement over whole animal in vivo experimentation. For the more impactful models such as the cuprizone model of demyelination we will only use full grown female mice and only feed them at a low concentration in pelleted feed to ensure the clinical signs are minimised. In addition, all drugs trialled to stop demyelination will be given orally in food or water so mice don't have to be injected daily and so the putative treatments will translate better into medical practice. Use of GA strains will increase the accuracy of our experiments; our GA mouse model of dementia shows no clinical effect.





194. Zebrafish models of human congenital eye malformations

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
 - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

zebrafish, eye formation, ocular malformations, CRISPR

Animal types	Life stages
Zebra fish	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our primary objective is to advance our understanding of the mechanisms controlling eye formation, and to identify new genes important for eye formation and involved in eye malformations 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?

Eye malformations are a main cause of childhood blindness and occur when genes controlling eye formation during pregnancy are defective. However, defective genes have only been identified in 30% of affected patients, meaning that in most cases, the genetic cause of the defects seen in these children are unknown. Often eye defects are only seen when several genes are simultaneously defective, making the identification of these genes



more difficult. One tool to identify them are animal models with a defect making them prone to develop eye malformations. Our primary objective is to generate genetically altered zebrafish strains to uncover new genes involved in eye malformations, using one of these animal models.

The data generated over the life of this project licence will lead to the identification of new genes involved in promoting human congenital eye malformations when disrupted. The inclusion of these new candidates in screening panels for ophthalmic conditions will expand the number of identified causes of congenital eye malformations, and improve our diagnosis approaches. Additionally, this programme of research will expand our knowledge of the genes involved in eye formation in normal conditions, an essential prerequisite to understand the mechanisms involved in eye disease. In the long term, this research will therefore benefit the society by improving diagnostic strategies and genetic counselling for the affected patients. Other researchers in our field will also benefit from our studies, which will build up our overall knowledge of eye formation.

What outputs do you think you will see at the end of this project?

Our research will identify new genes involved in promoting human congenital eye malformations when disrupted. Additionally, our research will expand our knowledge of the genes involved in eye formation in normal conditions, an essential prerequisite to understand the mechanisms involved in eye disease.

Who or what will benefit from these outputs, and how?

This project will identify new genes to be considered in the aetiology of human congenital eye malformations. Our studies generate new basic knowledge and have several main beneficiaries: other researchers in the field, affected patients with ophthalmic diseases, and the wider society.

In the short term, our studies will have an immediate impact on our overall knowledge of eye formation and mechanisms of embryonic development. The new genetically altered strains of zebrafish that we may generate will be made available to the wider research community, and will be useful tools not only for ourselves, but also for other researchers, to expand our understanding of the genes involved in eye formation.

Basic research findings may need between a few years and decades to reach clinical practice. The impact of our studies on better diagnosis may thus not be immediate. Despite this, our studies will be essential to inform clinical practice and we expect they will have a long term impact, beyond the life of the project. Indeed, genes identified in our studies will constitute new candidates to be included in screening panels for ophthalmic conditions. This will expand the number of identified causes of congenital eye malformations, improving our diagnosis approaches and genetic counselling for the affected patients.

How will you look to maximise the outputs of this work?

We maintain long term collaborations with other researchers in the field. As part of these collaborations we have also links with clinicians in the ophthalmology field, ensuring a route of communication of our basic research findings to the clinical practice. In addition, we share expertise and resources with other groups at our institution, where we form part of a well established group of researchers focused in understanding the cell biology and the genetics underlying a variety of human congenital diseases.



An essential part of scientific research is the communication of our studies to colleagues in the field. We will maximise the dissemination of our results by participating in national and international conferences in our field of research, where we will present our studies prior to publication. The results from our work will be subsequently published in peer reviewed, open access scientific journals. Our institution has signed the San Francisco Declaration of Research Assessment (DORA) and maintains a public access repository in which all our publications will be stored and made available to the wider research community. We actively participate in outreach events at our institution, and maintain an up to date institutional website in which the aims and results of our research are regularly updated.

Species and numbers of animals expected to be used

- Zebra fish (*Danio rerio*): 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will make use of the zebrafish as a model system. Our choice of animal model allows us keep the number of adult animals used to the minimum as compared to other vertebrates. Indeed, zebrafish embryos are fertilised externally and one pair of adult fish generates hundreds of embryos per week. This allows us to perform extensive phenotypic analysis in high numbers of embryos, without the need to sacrifice the mother. We have refined our approaches so that most of the procedures will generate embryos for analysis prior to 5 days post-fertilisation. This will ensure that we maintain at the minimum our use of mature animals. A pair of healthy breeding zebrafish adults produces over a hundred embryos per mating, providing us with substantial amount of material to perform embryonic manipulations.

Typically, what will be done to an animal used in your project?

We expect to generate several new GA zebrafish strains over the life of this project. Some of these lines will carry mutations in the candidate genes selected over the course of our pilot phenotypic screen, and others may carry an exogenous transgene (if for example we need to generate a new Gal4-expressing line for some of our manipulations). These lines will be kept in heterozygosis, and we expect no adverse effects to show in them. Adult animals will be bred to maintain the colonies of GAAs necessary for the progression of the project, and to obtain embryos for analysis. Adults are not expected to show any adverse effects, and will be kept alive in the facility up to 18 months of age.

We have refined our experimental approaches so that most of the procedures to be performed in the course of the project will require phenotypic analysis prior to 5 dpf and will thus not constitute regulated procedures. These embryos will be euthanised by using a schedule 1 method (when old enough to have developed a nervous system) and subsequently will be treated for tissue analysis. Occasionally, manipulated embryos may be grown beyond 5 dpf to perform visual acuity tests on them. These embryos will be subjected to anaesthesia to perform the analysis. We will keep these tests to the minimum, and the embryos will be euthanised by a schedule 1 method upon completion of the test.



What are the expected impacts and/or adverse effects for the animals during your project?

Regulated procedures in this project will involve generation of new genetically altered strains, genotyping of adult animals, or optokinetic response tests to assess visual acuity of larvae. Genetically altered strains of fish produced under this project are not expected to exhibit any harmful phenotype. Some fish may have the potential to develop a harmful phenotype after a certain age. Fish exhibiting any unexpected harmful phenotypes will be killed by a schedule 1 method, or in the case of individual fish of particular scientific interest, advice will be sought promptly from the assigned Home Office Inspector. The genotyping procedure in place is not expected to have a negative impact on the wellbeing of the fish subjected to it. Measures have been put in place to monitor and assess any unexpected adverse effects, and schedule 1 methods for euthanasia will be used in those cases where they are detected. Similar considerations have been taken in the case of the visual acuity test, which is not expected to result in any pain or suffering to the animals analysed. In all cases, the health and wellbeing of all adult fish will be regularly monitored and they will be euthanised by a schedule 1 method at the first sign of suffering. Protocols published by the Zebrafish International Resource Centre Health Service and the British and European legislation will be closely followed to monitor health status in our animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Only mild severity in a small proportion of animals (<5%) is expected to show at any given time. As detailed above, some fish may have the potential to develop a harmful phenotype after a certain age, and to avoid any suffering fish will be closely monitored for signs of disease and immediately euthanised if any is detected.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project outlined in this proposal aims at understanding how the eyes form during embryogenesis, and to search for genes that when affected lead to eye disease. The nature of this work requires the use of whole animals. Indeed, visualisation of the processes involved in eye formation can only be performed in an intact organism. In addition, the recapitulation of disease features in congenital eye malformations can only be accomplished in a whole animal. We will make use of the zebrafish as a model system. Our choice of animal model allows us keep the number of adult animals used to the minimum as compared to other vertebrates. Indeed, zebrafish embryos are fertilised externally and one pair of adult fish generates hundreds of embryos per week. This allows us to perform extensive phenotypic analysis in high numbers of embryos, without the need to sacrifice the mother.



Which non-animal alternatives did you consider for use in this project?

There are no in vitro alternatives available to recapitulate eye formation, since we need the intact organism, and the complex interaction of multiple tissue types, to generate a fully functional eye.

We have therefore done a detailed literature review and analysis of previous research to inform our project, and ensure that we optimise our approaches and our selection of candidate genes. This allows us to keep to the minimum the number of animals we need to use for our research.

Why were they not suitable?

The generation of a fully functional eye requires the complex interaction of multiple tissue types, and thus is currently not possible to use it as a substitute to animal work. Instead, the right choice of animal model and the careful review of previous research allows us to keep to the minimum the number of animals we use.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In the course of the project we will make use of several strains of fish with different modifications in their genome. The use of these organisms is essential for the progress of this project. Adult fish will only be maintained for the purpose of obtaining the embryos required for the research. Animals enough to maintain one generation of breeding adults per strain at any time will be kept. From previous experience, we estimate this number to be around 15 adult breeding animals. New generations will be grown when breeding adults reach 8/12 months of age and generations will be replaced every 18 months. Thus, at any given time we expect to have between 60 and 80 fish (between juveniles and adult breeding fish) per strain.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have refined our approaches so that most of the procedures will generate embryos for analysis prior to 5 days post-fertilisation. A pair of healthy breeding zebrafish adults produces over a hundred embryos per mating, providing us with substantial amount of material to perform embryonic manipulations. When derived from a strain bearing a mutation, pools of embryos comprising normal and affected embryos will be treated and analysed all together, ensuring a reduced bias and uniformity in the experimental procedures.

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 done a detailed literature review and analysis of previous research to inform our project, and to ensure that we optimise our approaches and our selection of candidate genes. This allows us to keep to the minimum the number of animals we need to use for our research.

Most of the experiments described here will be performed on zebrafish embryos not older than 5 days post-fertilisation. Adult fish will only be maintained for the purpose of obtaining the embryos required for the 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.

Most of the experiments described in this project licence will be performed on zebrafish embryos not older than 5 days post-fertilisation. Adult fish will only be maintained for the purpose of obtaining the embryos required for the research. Our choice of animal model allows us keep the number of adult animals used to a minimum as compared to other vertebrates. Indeed, zebrafish embryos are fertilised externally and one pair of adult fish generates hundreds of embryos per week.

Mild procedures may be performed on the fish, if genotyping of the adults is required. In these cases, animals subjected to a procedure will be provided with adequate analgesia and closely monitored for recovery. Once the animals are not needed anymore, they will be euthanised using an established procedure.

We expect to generate several new zebrafish strains with genetic modifications over the life of this project. These strains will be kept in heterozygosis, and we expect no adverse effects to show in them. In any case, any manipulated embryo allowed to grow over 5 days post-fertilisation will be closely monitored for signs of adverse effects.

Why can't you use animals that are less sentient?

We have refined our approaches so that most of the procedures will generate embryos for analysis prior to 5 days post-fertilisation. This will ensure that we maintain at the minimum our use of mature animals. A pair of healthy breeding zebrafish adults produces over a hundred embryos per mating, providing us with substantial amount of material to perform embryonic manipulations.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Adverse effects in our animals will be scored by looking at growth rates and swimming patterns. Any juveniles that show a significant difference in size to their siblings, or aberrant swimming patterns, will be immediately euthanised. The health and well-being of adult fish will be regularly monitored and they will be euthanised at the first sign of



suffering, following the protocols published by the Zebrafish International Resource Centre Health Service, and the British and European legislation.

Any animal subjected to mild procedures will be provided with adequate analgesia. The technical staff responsible for animal handling will be appropriately trained to ensure the best handling of the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

As detailed above, the protocols published by the Zebrafish International Resource Centre Health Service and the British and European legislation will be followed to provide the best care to our animals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will maintain a continued communication with the staff in our animal facility, and follow their advice and suggestions regarding replacement, reduction and refinement of our experimental approaches.

We will keep ourselves informed about any new advances by regularly consulting the NC3Rs website. As part of our dissemination strategy we participate in conferences and meetings in our field of research. Conferences such as the European Zebrafish Meeting, the European Principal Investigators Meeting and the International Zebrafish Conference allow us to keep up to date with the latest advances in zebrafish husbandry, welfare and research.





195. Zebrafish models of movement 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
- 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

No answer provided

Animal types	Life stages
Zebra fish	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 aim of this project is to obtain a better understanding of the mechanisms leading to Parkinson's disease (PD).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

PD is the second most common neurodegenerative disorder after Alzheimer's disease. in the UK alone, at least 120,000 people are affected. PD is relentlessly progressive and incurable. Symptomatic treatment only addresses some of the symptoms. The longer a patient with PD is on medication, the more likely it becomes for him to benefit less from the medication and develop more and more troublesome side effects. Genetic research has now identified ~ 90 inherited risk factors for PD. However, it is often unclear how these



genetic risk factors contribute to the risk of PD: Their biological role is often ill defined and it is also not known how they interact with each other. In addition, it is not known whether any of them make individuals more vulnerable to environmental PD toxins. It is also unclear how these PD risk genes interact with ageing-related mechanisms. Ageing is by far the biggest risk factor for neurodegenerative disorders but the link between PD and ageing is poorly understood. We and others have previously demonstrated the unique usefulness of zebrafish (*Danio rerio*) to study mechanisms linked to PD. We now want to continue our work by focussing on PD risk genes. We particularly want to focus on their interaction over time and how these PD risk genes interact with ageing-related mechanisms.

What outputs do you think you will see at the end of this project?

We expect our research to provide crucial new insight into the mechanistic role of PD risk genes. We anticipate that the use of the zebrafish model will be particularly helpful to study the interaction between PD risk genes. We will particularly focus on "druggable" mechanisms such as lysosomal dysfunction, inflammation or mitochondrial dysfunction. We also expect important insight from our studies on the interaction between PD risk genes and ageing-related mechanisms. Our research will hopefully help towards a better understanding of the mechanisms leading to PD and provide novel insights for future disease-modifying therapy with particular focus on personalized medicine and disease modification.

We will present key findings of our research at local, national and international scientific meetings. We will also publish our findings in peer-reviewed scientific journals. We will share our results with relevant PD charities in general and both local and regional PPI groups in particular.

Who or what will benefit from these outputs, and how?

Short-term benefits (within the 5 yr currency of this license):

Our MRC-funded on-going work has provided us with the opportunity to make PD GWAS mutant lines and begin their functional characterisation. We have already undertaken and published a detailed characterisation of a *gba*^{-/-} mutant zebrafish line which confirmed early inflammation preceding neuronal cell loss and alpha-synuclein independent loss of dopaminergic neurons. We subsequently undertook gene-gene interaction studies to clarify the mechanistic role of miR155 for neuronal cell death in GCase deficiency, again the results were published in a respected Neuroscience journal. We have now also nearly completed the characterisation of a *gch1*^{-/-} zebrafish line, GCH1 being a further PD risk gene. We've identified marked pro-inflammatory changes in the *gch1*^{-/-} embryos/larvae. This is an unexpected finding which might indicate that anti-inflammatory medication in patients with GCH1 risk variants may benefit from anti-inflammatory medication. Over the next 5 years, we are hoping to study further PD GWAS risk lines in great detail. Our initial focus will be on *gak*, *tmem175*, *mccc1*, and *vps13c* mutant lines. We hope to gain insight into which "druggable" mechanisms (ie lysosomal function vs mitochondrial function vs neuroinflammation) are affected in these PD GWAS mutant lines. Any PD risk gene only confers a small risk. We will therefore also cross different mutant lines with an assumed shared/additive mechanism to confirm or refute the hypothesized interaction.

Expected timescale: We expect to publish the detailed characterisation of at least one novel PD GWAS mutant zebrafish line every year over the next five years. We expect to



publish at least two gene-gene interaction studies towards the second half of the 5 yr currency of this license.

Ageing is the single most important risk factor for all neurodegenerative diseases. Our proposed approach of crossing PD GWAS mutant lines with senescence mutant zebrafish lines will allow us to study the interaction of distinct ageing-related mechanisms such as impaired DNA repair with mechanisms specific for the respective PD GWAS mutant line.

Expected timescale: We expect to publish at least three manuscripts describing the effect of different senescence mutant lines (klotho, ercc1, polg1) during the 5 yr currency of this license. The proposed studies are complex, we therefore only expect to publish the first study in 2 yr.

We hope that the detailed mechanistic characterisation of PD GWAS mutant lines and their interaction with ageing-related mechanisms will provide distinct "druggable" targets. Where appropriate and permissible within this license, we will aim to undertake focussed rescue experiments, typically using already FDA licensed compounds with a proven or putative effect on the respective targets.

Expected timeline: We expect to include the testing of at least one compound in all our publications (see above).

Non-scientific benefits: Patients and their families find it extremely re-assuring to learn about our research. This is particularly the case for patients and families from our region (South Yorkshire).

Medium-long term benefits:

Our work will contribute to a better understanding of mechanisms leading to PD. It is this improved understanding which will hopefully lead to better treatment for PD and related disorders. Insight provided from our work will hopefully help towards the development of "Precision Medicine" approaches for PD.

How will you look to maximise the outputs of this work?

We are very keen to collaborate widely and already doing so with other local, national and international scientists. Where appropriate, we will use press releases to disseminate our new knowledge. The publication of negative data is crucial and we are absolutely committed to it. We will follow the ARRIVE guidelines when disseminating our work. We will also, wherever appropriate, make all data freely available, for example by depositing them in biological data repositories.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): Up to 60,000 zebrafish over a 5 year period.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.



Zebrafish are vertebrates and therefore more closely related to humans than worms or flies. We would like to use zebrafish to get a better understanding of Parkinson's disease (PD). PD is the second most common neurodegenerative condition after Alzheimer's disease. In the UK alone, > 120,000 people are affected. PD is relentlessly progressive and incurable. Better understanding of the underlying mechanisms to PD will hopefully lead to improved therapy for patients with this condition. At least 90 genes contribute to the risk of an individual to develop PD but the role of most of these genes is poorly understood. The zebrafish genome is very similar to the human genome and it is now technically easy to make changes in zebrafish genes which cause PD. This allows us to study the function of these genes in a living organism. PD is typically a late onset condition but the disease process can already start early. It is therefore important that we can study the effect of PD genes throughout the entire lifespan of the PD gene mutant zebrafish lines.

Typically, what will be done to an animal used in your project?

The vast majority of any invasive experiments will only be undertaken before the zebrafish larvae become protected animals (before 5.2 days post fertilization). The only surgical procedure in fish older than 5.2 days past fertilization is fin-clipping to enable genotyping. We will not undertake any other invasive procedures such as injections into fish older than 5.2 days post fertilization either. We will only administer compounds to explore their therapeutic potential after careful literature review and subsequent to dose finding experiments in WT embryos/larvae. We nevertheless acknowledge the potential for toxicity.

What are the expected impacts and/or adverse effects for the animals during your project?

PD mutant zebrafish may develop slowness of movement, similar to the symptoms observed in PD patients. Weight loss may be observed as part of general malaise. We do not expect any PD gene mutant zebrafish to develop tumours or 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)?

We anticipate there may be a mild to moderate impairment of the wellbeing of the zebrafish disease models, however once their condition begins to deteriorate rapidly we will sacrifice them humanely.

Some of the mutant zebrafish lines may have dominantly or recessively inherited neurological phenotypes manifesting as a swimming deficit. In dominant models 50% will be affected, in recessive models 25% will be affected.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 characterise molecular mechanisms of neurological diseases we must perform some experiments at the level of the whole organism. We use cells and tissue samples where it is possible to do so, but ultimately we need to understand how neurons die in their natural context. Neurons are highly specialised cells, which interact with a wide variety of other cell types both inside and outside the brain and spinal cord. Each interaction has its own complicated chemical and physical signals. Such complexity is impossible to replicate in culture systems. The use of animals will also allow us to study the effect of ageing and the interaction of ageing with PD genes. Ageing is by far the most important risk factor for PD, but the underlying mechanisms and how ageing interacts with PD genes is poorly understood.

Which non-animal alternatives did you consider for use in this project?

We are already using the most advanced, state-of-the-art cell culture models for PD, namely fibroblast-derived inducible nerve cells (i-neurons). However, it is impossible to study the effect of PD genes on different cell types (such as neurons, immune competent cells and glial cells) in a cell culture system.

Why were they not suitable?

Cell culture systems do not allow us to study the interaction between different cells in the same way this is possible in a living animal. It is also not possible to study the effect of ageing in cell culture systems to the same extent we can study ageing in an animal model.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate that this is the maximum number of animals we will need to use in order to meet our objectives over a 5 year period.

Power calculations are used to determine the number of zebrafish required for each experiment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We carefully design our experiments to minimise variability and this allows the use of the smallest number of animals required to produce statistically significant results. The experimental approaches we use have been discussed with statisticians and academic experts in experimental design.



It is important to use an appropriate number of animals for each experiment, that is not too large, as this would be wasteful, or too small as this may not provide statistically significant results.

We base the number of animals to be used for each experiment on our previous experience. This may be from similar studies using the same PD mutant zebrafish model, or on pilot studies used to look for the variability in the data we will obtain, and the size of the difference observed between control and PD mutant zebrafish.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Zebrafish are highly efficient breeders, so we are able to obtain large numbers of embryos <5.2dpf from a small number of parents. We will use pilot studies to optimise the number of animals used in this project. We will share tissue where it is feasible to do so.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use zebrafish models of human neurological disease because they offer several technical advantages compared to alternative species such as mice for our experiments.

Fish are vertebrates (i.e. their brain is in many ways similar to the human brain) so represent a simple yet appropriate model for studying human neurological diseases. The models themselves are generally mild, but occasionally moderate severity. On balance, this outweighs the unmet need for scientific advance in order to identify therapeutics.

We will use ZEG analysis where applicable to ensure that we only raise those fish that are required for adult experiments.

Why can't you use animals that are less sentient?

The majority of animals used under this license will be embryos <5.2 days old. These are not considered protected animals for the purposes of ASPA, thus we already use the least sentient model. Where we do use life stages >5.2 days old we believe that zebrafish are less sentient than mice, which would be the most suitable alternative model system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Sometimes we require single housing of zebrafish, and where we don't think this will jeopardise the welfare of the fish we will use companion animals.



We use close monitoring of adult zebrafish disease models using score sheets to monitor levels of distress. In the event that genetically altered zebrafish shows any distress, for example caused by abnormal swimming, this allows us to implement a humane endpoint.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE guidelines to ensure that experiments are conducted in the most refined way and where applicable we will use the NC3Rs Experimental Design Assistant to aid our studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will follow the relevant literature for experimental design and attend relevant meetings.

To implement changes I will work with my research group to pilot new approaches where we believe these may improve 3Rs aspects of our work.

I will consult with our NC3Rs regional programme manager to keep abreast of the latest developments, e.g. Fish Behaviour Index, <https://www.nature.com/articles/s41598-019-45464-w>



196. Neuropharmacology of vulnerability to compulsivity

Key Words

Addiction, compulsivity, vulnerability, neuropharmacology, rat

Expected duration of the project

5 years

Purpose of the project (as in Article 5)1

- Basic research
- Translational and applied research

Describe the objectives of the project (e.g. the scientific unknowns or scientific/clinical needs being addressed)

Our research aims to identify the environmental, behavioural, neurophysiological, cellular and molecular substrates of individual vulnerability to develop impulsive / compulsive disorders such as drug addiction or Obsessive Compulsive Disorders to eventually develop new treatments for the people suffering from these disorders. This research project aims at providing strong proof of concept of the contribution of a specific mechanism or the effectiveness of a specific drug for translating into human studies.

What are the potential benefits likely to derive from this project (how science could be advanced or humans or animals could benefit from the project)?

Impulsive / compulsive disorders cost the UK an estimated £32 billion per year. Thus, these neuropsychiatric disorders, that include obsessive / compulsive disorder, drug addiction, or dopaminergic dysregulation syndrome place a considerable burden on not only the affected individual, but also social and economic burdens on society. Our research will provide new insights into the behavioural, neural and cellular substrates whereby some individuals are vulnerable to these disorders and open new avenues for the development of new treatments for them.

What species and approximate numbers of animals do you expect to use over what period of time?

We use behavioural models in rats. We use the minimum numbers of animals possible to achieve biologically and statistically meaningful data. We anticipate that we will use fewer than 5350 over 5 years.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

For the majority of our animals (85%), we anticipate no more than transient discomfort and no lasting harm. However, as some of the disorders we study cause distress in humans (e.g. obsessive / compulsive disorder, Parkinson Disease, or drug addiction) then some of our animals, namely those that are the most vulnerable to develop



neuropsychiatric disorders (~15%), do experience more stressful conditions (including repeated exposure to stressors such as mild footshocks, loss of weight) (of no more than moderate severity) which reflect the spontaneous propensity to develop an aberrant behavior in the face of specific environmental conditions (such as drug exposure, frustration, stress). In some of our experiments, we have to manipulate the brain (e.g. by surgically damaging specific regions) in order to understand why and where neuropsychiatric disorders happen or to insert vascular cannulae to allow animals to self-administer non ingestive drugs. The development of compulsive behavior or causal manipulations of the brain could both cause moderate severity. When we need to do these procedures, the animals are very carefully monitored for any signs of pain or distress. If the animals show signs of suffering and we are not able to ameliorate these very rapidly in consultation with the named veterinary surgeon, then we euthanize the animal. Fortunately, such instances are very rare.

The study of interoceptive mechanisms in impulsive and compulsive disorders relies on drug discrimination tasks that requires repeated, often between 40 and 50 intraperitoneal injections. Distress and potential severity related to these procedures are minimized by performing the injections without restraining the animals, using the smallest needles, and volume of drug to be administered (pH neutral) as well as the lowest pressure during injection. Animals are also closely assessed every batch of 24 injections by an NVS. At the end of experiments, the animals are killed.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives

This research is only possible with the use of animals because human studies (e.g. brain imaging studies) are useful, but can only provide correlative data that do not address causation. Furthermore, it is not ethically possible to study the genetic and/or environmental factors that underlie predisposition to, and the development of, neuropsychiatric disorders in humans. Similarly, it would not be possible to develop new treatments for brain disorders without testing them in animal models first. In vitro models (e.g. brain slice preparations) or computer simulations cannot be used because the modelling of behaviour in these systems is not sufficiently advanced.

Reduction

Explain how you will assure the use of minimum numbers of animals

We are fully committed to using the minimum number of animals required to obtain data that are statistically and biologically meaningful. We carefully design our experiments to maximise the behavioural data collected from each animal, and to minimise distress.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

We use rats because they are the least sentient species which allow researchers to investigate marked inter-individual differences in sophisticated behaviours that capture core features of the neuropsychiatric disorders we are interested in. Additionally, the brain



circuitry implicated in many neuropsychiatric disorders is highly conserved between rodents and humans. We take the welfare of the animals very seriously. Most of our animals run in long-lasting behavioural experiments in which they perform tasks for food or drug reward, and experience procedures, e.g. injections that produce only transient discomfort and no lasting harm, achieved by a constant refinement of administration techniques by well-trained personnel. Animals are monitored frequently (often undergoing daily testing). In the case of drug self-administration procedures, the daily monitoring of the status of the port area is facilitated by a severity scale developed in collaboration with previous NVSs that is used as a visual reference by all members of staff to monitor the animals. Any adverse effects are discussed with the named veterinary surgeon. If these cannot be quickly ameliorated then animals are euthanized to prevent suffering